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14. ABSTRACT The research conducted in this COE grant is based on the paradigm that estrogens can become endogenous carcinogens when their metabolism is unbalanced, favoring formation of catechol estrogen quinones and their reaction with DNA. Compelling evidence obtained in the various specific aims of this COE will be decisive for determining the risk of breast cancer by using the depurinating estrogen-DNA adducts as biomarkers. These biomarkers will also be used for evaluating the ability of specific antioxidants to prevent breast cancer initiation. Much of the research accomplished by this COE was published in a review article in BBA-Reviews in Cancer, which was co-authored by all the participants of the COE.

15. SUBJECT TERMS Estrogens, estrogen-initiated cancer, depurinating estrogen-DNA adducts, analysis of estrogen metabolites and conjugates, estrogen-induced mutation and cell transformation, overexpression of aromatase, gene expression by DNA microarray, human breast ductal lavage, breast cancer prevention, biomarkers for breast cancer susceptibility

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SUMMARY OF OVERALL DISCOVERIES

The research conducted in this COE grant was based on the paradigm that estrogens can become endogenous carcinogens when their metabolism is unbalanced, favoring formation of catechol estrogen quinones and their reaction with DNA [1]. The further evidence obtained in the various specific aims of this COE will be decisive for determining the risk of breast cancer by using the depurinating estrogen-DNA adducts as biomarkers. These biomarkers are also being used for evaluating the ability of specific antioxidants to prevent breast cancer initiation.

Much of the research accomplished by this COE was published in a review article in *BBA-Reviews in Cancer* [1], which was authored by all of the participants in the COE.

In Specific Aim 1, the major discovery has been to introduce a new methodology for analyzing 40 estrogen metabolites, estrogen conjugates and depurinating estrogen-DNA adducts. This new methodology has enabled us to determine that healthy women have relatively low levels of estrogen-DNA adducts, compared to women at high risk of breast cancer and women with breast cancer, who have relatively high levels of these adducts ($p<0.001$). The relatively high levels of estrogen-DNA adducts in women with breast cancer or at high risk for the disease have been detected in three studies, two with urine samples [2,3] and one with serum samples [4]. This new finding has led to studies of prevention with specific antioxidants that decrease the level of depurinating estrogen-DNA adducts, which represent the first critical step in the initiation of breast cancer.

In Specific Aim 2, we demonstrated that the human breast epithelial cells (MCF-10F cells), which are estrogen receptor-negative, are transformed at physiological doses of estradiol and 4-hydroxyestradiol and to a much lesser extent by 2-hydroxyestradiol. These transformations also occur in the presence of antiestrogens, such as ICI-182,780. Furthermore, selected cells from these transformed populations form solid tumors in SCID mice. These results support the hypothesis that initiation of breast cancer occurs through the genotoxicity of estrogens.

In Specific Aim 3, the major goal has been to demonstrate that 4-hydroxyestradiol is mutagenic, in both cultured BB rat2 embryonic cells and the mammary gland of female Big Blue rats. Once again, the mutations were observed after treatment with 4-hydroxyestradiol, but not 2-hydroxyestradiol, suggesting that the major culprit in initiation of cancer by estrogens derives from 4-catechol estrogens and not 2-catechol estrogens. These results are analogous to those found in the cell transformation studies in Specific Aim 2.

The studies in Specific Aim 4 have been conducted with female ERKO/Wnt-1 mice, which develop mammary tumors, but do not express estrogen receptor-alpha. Using ovariectomized ERKO/Wnt-1 mice, in a dose-response experiment it was demonstrated that the development of mammary tumors was proportional to the dose of estradiol implanted in the mice. These studies suggest that genotoxic pathways of estrogen carcinogenesis play the critical role in the initiation of these mammary tumors and that aromatase inhibitors should be superior to antiestrogens for the prevention of breast cancer.

The work conducted in Specific Aim 5 contributed to the clinical study in Specific 1, in which analysis of estrogen compounds in urine samples from 12 women at high risk for breast cancer and 17 women with a personal history of breast cancer was compared to the analysis of urine samples from 46 healthy women. The results of these analyses demonstrated that the relative levels of depurinating estrogen-DNA adducts were significantly higher in urine samples from women with breast cancer ($p<0.001$) or at high risk ($p<0.001$), compared to control subjects. The high-risk and breast cancer groups were not significantly different ($p=0.62$). These results support the hypothesis that depurinating estrogen-DNA adducts can serve as potential biomarkers of risk of developing breast cancer.

The Molecular Biology Core continued to study the role of cytochrome P450 1B1 in estrogen hydroxylation and risk of developing breast cancer. This Core contributed to the areas of comparative metabolism, aromatase inhibition, genetic predisposition and tumor classification. In genomic analysis of the cell transformation model established by the Russo laboratory, the Core identified important molecular events leading to the expression of tumorigenic markers and epithelial-mesenchymal transition, which is an important cellular determinant of invasiveness and metastasis. By identifying these characteristics occurring during malignant cell transformation by estrogens in the estrogen receptor-negative cell line MCF-10F, it identifies a new and essential cell model for understanding the especially aggressive characteristics of estrogen receptor-negative tumors.

The Analytical Core supported the studies in Specific Aims 1-5 by analyzing samples from human urine, mouse mammary tissue, rat mammary tissue and culture medium from MCF-10F cells. Ultraperformance liquid chromatography/tandem mass spectrometry was used to identify and quantify estrogen metabolites, conjugates and/or depurinating DNA adducts in these samples.

The Advocacy Core served as an integral part of the COE, providing input into the specific aims of the grant, as well as specific advocacy issues related to consent documents, pilot study design and implementation and funding opportunities. The Core published a consumer guide to involvement in basic research, entitled “Partners in Research, Advocates & Scientists: Advocates’ Guide”. This guide is available for upload to organizational websites.

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SPECIFIC AIM 1 – CAVALIERI

A. Introduction

The basic hypothesis guiding this research is that endogenous estrogens can be oxidatively metabolized to catechol estrogen quinones that react with DNA to form specific DNA adducts to generate tumor-initiating mutations. These DNA adducts are potential biomarkers for breast cancer and risk of developing breast cancer. In fact, the level of the 4-hydroxyestrone(estradiol)-1-N3Adenine adduct [4-OHE$_1$(E_2)-1-N3Ade] has been observed to be significantly higher in urine from men with prostate cancer or other urological conditions than in urine from healthy control men, in which the adduct is at background levels [1]. The results obtained in animal models, cell culture and human breast tissue led us to select several compounds to prevent the genotoxicity of estrogens that we think is at the origin of breast cancer. The selected compounds target different steps involved in the mechanism of tumor initiation. Prevention studies will demonstrate that estrogen genotoxicity plays a critical role in the initiation of breast cancer. In addition, the results will lay the groundwork for designing a clinical research study of breast cancer prevention and developing bioassays for susceptibility to this disease. With these goals in mind, we have obtained data demonstrating the utility of the depurinating estrogen-DNA adducts as biomarkers for risk of developing breast cancer and for use in prevention studies..

B. Body

B-i. Methods and Procedures

Research was previously completed on Specific Aim 1a.

In Specific Aim 1b, we continued analyzing urine from women with and without breast cancer by using ultraperformance liquid chromatography/tandem mass spectrometry (UPLC/MS/MS) and began analyzing serum samples.

B-ii. Results

Specific aim #1a: Investigate the prevention of estradiol (E_2)-induced tumors in the mammary gland of female ACI rats by analyzing the profiles of estrogen metabolites, conjugates and depurinating DNA adducts in treated animals at various time-points and the development of tumors in the animals.

No further research was conducted on this specific aim because (1) the experiments were completed, (2) we realized that the planned experiments would not yield useful results, and (3) we had found that we could conduct more informative studies in humans.

Specific Aim #1b: Analyze the profiles of estrogen metabolites, conjugates and depurinating DNA adducts in ductal lavage samples from women with and without breast cancer.

In our initial study (not in this grant) we found that men with prostate cancer or other urological conditions had high levels of the estrogen-DNA adducts in their urine, while the healthy control men had background levels of the adducts [1]. This was the first demonstration of a quantifiable relationship between formation of depurinating estrogen-DNA adducts and human cancer. The success of this study led us to undertake in the COE a broader study of estrogen metabolism and formation of adducts in women, based on the knowledge that adducts formed in tissues are excreted in urine. The estrogen metabolites, conjugates and depurinating

DNA adducts in spot urine samples (~50 ml) from 46 healthy control women, 12 women at high risk of breast cancer (Gail model score > 1.66%) and 17 women with breast cancer were preconcentrated from 2-ml aliquots by solid-phase extraction (SPE) and analyzed by ultraperformance liquid chromatography/tandem mass spectrometry (UPLC/MS/MS) [2]. The urine samples from healthy control women were provided by Dr. Paola Muti (under a different IRB protocol) or obtained at UNMC. Those from high-risk women were provided by Dr. James Ingle at the Mayo Clinic, and the ones from women with breast cancer were collected at the Mayo Clinic and at UNMC. The ratio of the adducts to their respective estrogen metabolites and conjugates was significantly higher in urine from the women with breast cancer ($p<0.001$) and high-risk women ($p<0.001$) than in urine from the healthy control women (Fig. 1) [2]. The levels in high-risk women and those with breast cancer did not differ ($p=0.62$). This study provided the first data showing that elevation of these DNA adducts is associated with high risk of developing breast cancer.

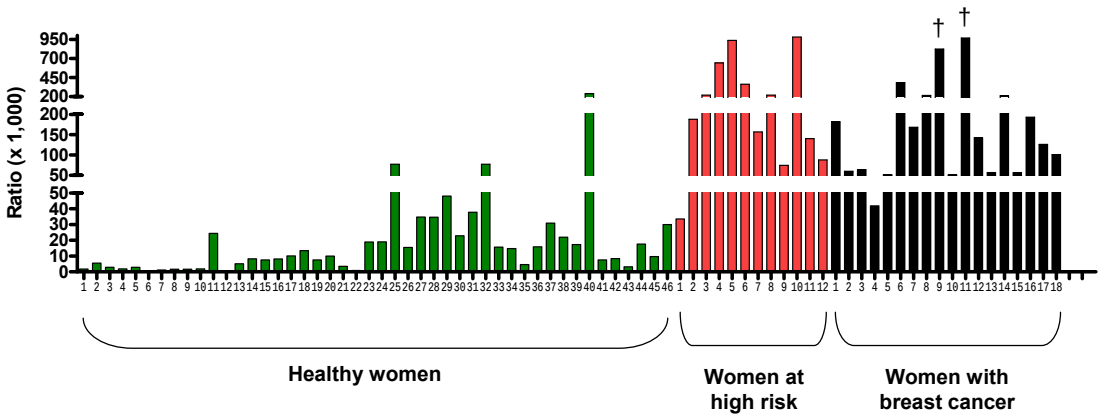

Figure 1. Depurinating estrogen-DNA adducts in the urine of healthy women, high-risk women and women with breast cancer [2]. The ordinate of this bar graph corresponds to the ratio of depurinating DNA adducts divided by their respective estrogen metabolites and conjugates:

$$\left(\frac{\text{4-OHE}_1(\text{E}_2)\text{-1-N3Ade + 4-OHE}_1(\text{E}_2)\text{-1-N7Gua}}{\text{4-catechol estrogens + 4-catechol estrogen conjugates}} + \frac{\text{2-OHE}_1(\text{E}_2)\text{-6-N3Ade}}{\text{2-catechol estrogens + 2-catechol estrogen conjugates}} \right) \text{x } 1000$$

†These are two urine samples from the same subject, collected 11 weeks apart.

We have repeated this study with 40 control women, 40 at high risk for breast cancer and 40 women with newly diagnosed breast cancer, all patients at the Mayo Clinic. The results of the first study were confirmed by analysis of urine (Fig. 2) and serum (Fig. 3) samples from these women. These results have been submitted for publication [3,4].

These results suggest that the estrogen-DNA adducts can be used as biomarkers for breast cancer. If the formation of estrogen-DNA adducts is the first critical event in the initiation of breast cancer, as we hypothesize, then these adducts could be used as biomarkers for early diagnosis of breast cancer risk.

These adducts also serve as surrogate endpoint biomarkers in studies of potential agents to prevent breast cancer. In fact, in an initial study with five healthy subjects (funded elsewhere), we have found that daily ingestion of 600 mg of the dietary supplement *N*-acetylcysteine for 30 days results in a significant decrease in the levels of estrogen-DNA adducts in urine and a concomitant increase in methoxy catechol estrogens, which represent the most

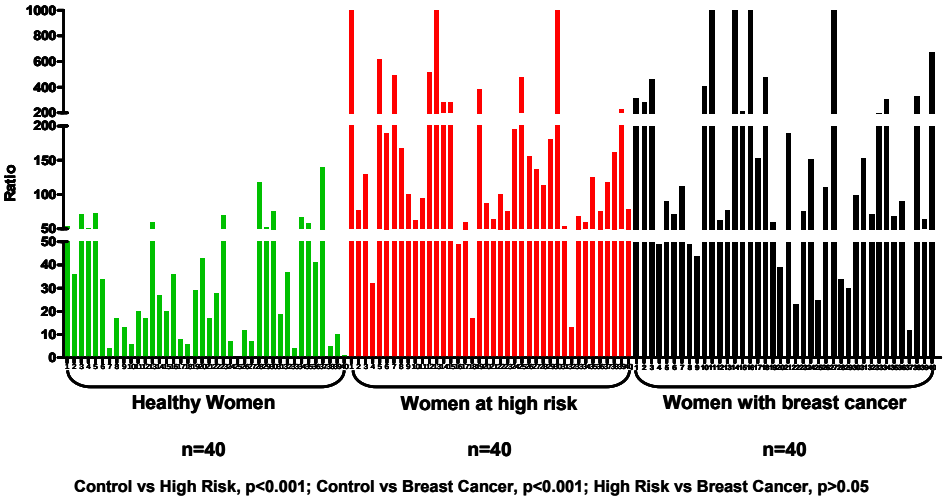

Figure 2. Depurinating estrogen-DNA adducts in urine of healthy women, high-risk women and women with breast cancer - second study [3]. These subjects are the same as those in Figure 3 nd are presented in the same order.

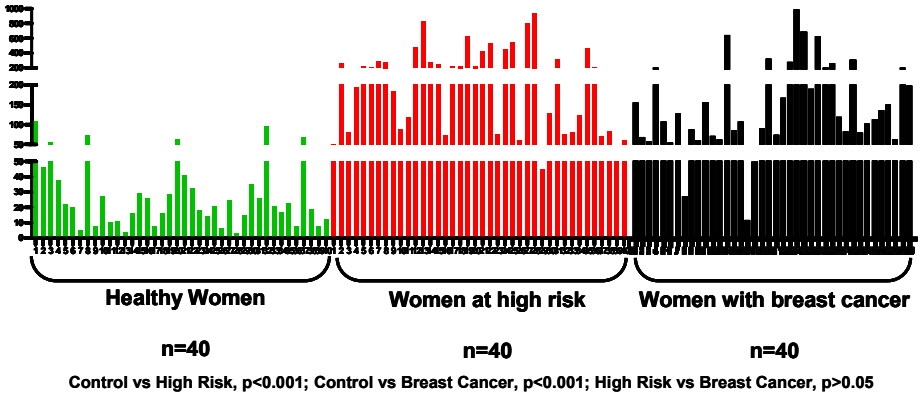

Figure 3. Depurinating estrogen-DNA adducts in serum of healthy women, high-risk women and women with breast cancer [4]. These subjects are the same as those in Figure 2 nd are presented in the same order.

important protective pathway in estrogen metabolism. These results provide preliminary evidence for one of the areas under study in the COE, the use of selected dietary supplements to reduce estrogen-DNA adduct formation and, presumably, reduce the risk of developing breast cancer.

C. Key Research Accomplishments

1. We have concluded that the Sprague-Dawley rat is not a model for estrogen-initiated tumors.
2. We have found in a small set of samples that nipple aspirate fluid from women with breast cancer contains estrogen metabolites, conjugates and depurinating DNA adducts, but nipple aspirate fluid from healthy control women does not contain the DNA adducts at levels detectable by UPLC/MS/MS. The adducts 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua in nipple aspirate fluid are potential biomarkers for breast cancer.
3. Most importantly, we have demonstrated that urine and serum from women with breast cancer and women at high risk of breast cancer (Gail Model score >1.66%) contain significantly higher levels of 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua than does urine from healthy control women. The adducts 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-

1-N7Gua in urine are potential biomarkers for risk of developing breast cancer and for demonstrating the efficacy of possible agents to prevent breast cancer.

D. Reportable Outcomes

a. Publications

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c. Invited Presentations and Abstracts

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E. Conclusions

1. We have not been successful in finding an animal model for estrogen-initiated mammary tumors.

2. We have detected estrogen metabolites, GSH conjugates and depurinating DNA adducts in our initial studies of nipple aspirate fluid. The adducts, 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua, were detected in nipple aspirate fluid from women with breast cancer, but not from women without breast cancer.

3. We have detected estrogen metabolites, GSH conjugates and depurinating DNA adducts in spot urine samples. The adducts, 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua, were detected at significantly higher levels in urine from women with breast cancer and women at high risk of breast cancer (Gail Model score >1.66%), compared to urine from women without breast cancer (Fig. 1) [2]. These adducts are potential biomarkers for risk of developing breast cancer and for demonstrating the efficacy of possible agents to prevent breast cancer.

4. We have detected estrogen metabolites, GSH conjugates and depurinating DNA adducts in our initial studies of serum. The adducts, 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua, were detected at baseline levels in serum from women without breast cancer. These adducts are potential biomarkers for breast cancer.

5. In our hypothesis, estrogen-3,4-quinones are initiators not only of breast cancer, but also of other types of human cancer, for example, prostate, non-Hodgkin's lymphoma, ovary, etc. Therefore, we hypothesize that higher levels of estrogen-DNA adducts in urine would be biomarkers for all of these cancers. Detection of depurinating estrogen-DNA adducts in nipple aspirate fluid would be a specific test for breast cancer.

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The molecular etiology of breast cancer: Evidence from biomarkers of risk

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Estrogens can become endogenous carcinogens *via* formation of catechol estrogen quinones, which react with DNA to form specific depurinating estrogen-DNA adducts. The mutations resulting from these adducts can lead to cell transformation and the initiation of breast cancer. Estrogen metabolites, conjugates and depurinating DNA adducts in urine samples from 46 healthy control women, 12 high-risk women and 17 women with breast cancer were analyzed. The estrogen metabolites, conjugates and depurinating DNA adducts were identified and quantified by using ultra-performance liquid chromatography/tandem mass spectrometry. The levels of the ratios of depurinating DNA adducts to their respective estrogen metabolites and conjugates were significantly higher in high-risk women ($p < 0.001$) and women with breast cancer ($p < 0.001$) than in control subjects. The high-risk and breast cancer groups were not significantly different ($p = 0.62$). After adjusting for patient characteristics, these ratios were still significantly associated with health status. Thus, the depurinating estrogen-DNA adducts are possible biomarkers for early detection of breast cancer risk and response to preventive treatment.

Key words: breast cancer risk; depurinating estrogen-DNA adducts; estrogen biomarkers; balance in estrogen metabolism

Development of noninvasive tests of breast cancer risk has been a major goal for more than 30 years. In this article we present biomarkers of risk that are related to the hypothesized first critical step in the initiation of breast cancer, namely, the reaction of catechol estrogen quinone metabolites with DNA.[1] Prevention of cancer can be achieved by blocking this DNA damage, which generates the mutations leading to the initiation, promotion and progression of cancer.[2]

Exposure to estrogens is a known risk factor for breast cancer.[3,4] The discovery that specific oxidative metabolites of estrogens, namely, catechol estrogen quinones, can react with DNA[5–9] led to and supports the hypothesis that these metabolites can become endogenous chemical carcinogens. Some of the mutations generated by this specific DNA damage can result in the initiation of cancer.[1,5] This paradigm suggests that specific, critical mutations generate abnormal cell proliferation leading to cancer.[1,10–13]

As illustrated in Figure 1, in the metabolism of catechol estrogens there are activating pathways[14] that lead to the formation of the estrogen quinones, estrone (estradiol) quinones [$E_1(E_2)$-Q], which can react with DNA. There are also deactivating pathways that limit formation of the quinones and/or prevent their reaction with DNA. These are methylation of catechol estrogens,[15] conjugation of the $E_1(E_2)$-Q with glutathione (GSH)[16] and reduction of the quinones to catechols[17] (Fig. 1).

When $E_1(E_2)$-3,4-Q react with DNA, they form predominantly the depurinating adducts 4-hydroxyestrone(estradiol)-1-N3Adenine [4-$OHE_1(E_2)$-1-N3Ade] and 4-hydroxyestrone(estradiol)-1-N7Guanine [4-$OHE_1(E_2)$-1-N7Gua],[5–7] whereas $E_1(E_2)$-2,3-Q form much lower levels of 2-hydroxyestrone(estradiol)-6-N3Adenine [2-$OHE_1(E_2)$-6-N3Ade] (Figs. 1 and 2).[7] Both $E_1(E_2)$-3,4-Q and $E_1(E_2)$-2,3-Q form much lower levels of stable DNA adducts than depurinating adducts.[5–7] Once released from the DNA, the depurinating estrogen-DNA adducts are shed from cells into the bloodstream and, eventually, are excreted in urine.

The release of the depurinating adducts generates apurinic sites in DNA, which in turn, may induce mutations. The observation of Harvey-*ras* mutations within 6–12 hr after treatment of mouse skin or rat mammary glands with E_2-3,4-Q suggests that these mutations arise *via* error-prone base excision repair.[1,10,11] Similar patterns of mutations have also been observed in the big blue (BB) rat mammary gland and cultured BB rat2 embryonic cells after treatment with 4-hydroxyestradiol (4-OHE_2) or E_2-3,4-Q.[1,12] The transforming activity of E_2 and 4-OHE_2 has been observed in human breast epithelial (MCF-10F) cells, which do not contain estrogen receptor-α, and it is not affected by the presence of an anti-estrogen.[18–20] Furthermore, 4-$OHE_1(E_2)$ are carcinogenic in the Syrian golden hamster and CD-1 mouse.[21–24] All of these results support the hypothesis that estrogens initiate cancer through their genotoxicity.

Initiation of cancer by estrogens is based on estrogen metabolism in which the homeostatic balance between activating and deactivating pathways is disrupted (Fig. 1). Activating pathways are the ones that oxidize E_1 and E_2 to their catechol estrogen quinones, whereas the deactivating pathways are the ones that block oxidation.[1] A variety of factors, such as diet, environment and lifestyle, can unbalance the equilibrium between these 2 pathways. When estrogen metabolism is balanced, the level of estrogen-DNA adducts in tissue and urine is low and/or the levels of estrogen metabolites and conjugates are high. In contrast, when estrogen metabolism is unbalanced, the level of DNA adducts in tissue and urine is high and/or the levels of estrogen metabolites and conjugates are low. It is this imbalance in estrogen metabolism, leading to relatively high levels of estrogen-DNA adducts, that may be a critical determinant of breast cancer initiation.

The above considerations led us to hypothesize that estrogen metabolites, conjugates and depurinating DNA adducts may differ between healthy women and women with breast cancer or at high risk of breast cancer. To test this hypothesis, we conducted a cross-sectional study in which 40 estrogen metabolites, conjugates and depurinating DNA adducts were analyzed in urine samples from healthy women, women at high risk for breast cancer based

Abbreviations: Cys, cysteine; ESI, electrospray ionization; $E_1(E_2)$-Q, estrone(estradiol)-quinones; GSH, glutathione; 4-OHE_2, 4-hydroxyestradiol; 4-$OHE_1(E_2)$-1-N3Ade, 4-hydroxyestrone(estradiol)-1-N3Adenine; 4-$OHE_1(E_2)$-1-N7Gua, 4-hydroxyestrone(estradiol)-1-N7Guanine; 2-$OHE_1(E_2)$-6-N3Ade, 2-hydroxyestrone(estradiol)-6-N3Adenine; MRM, multiple reaction monitoring; NAcCys, *N*-acetylcysteine; NI, negative ion; PI, positive ion; SPE, solid-phase extraction; UPLC/MS-MS, ultraperformance liquid chromatography/tandem mass spectrometry.

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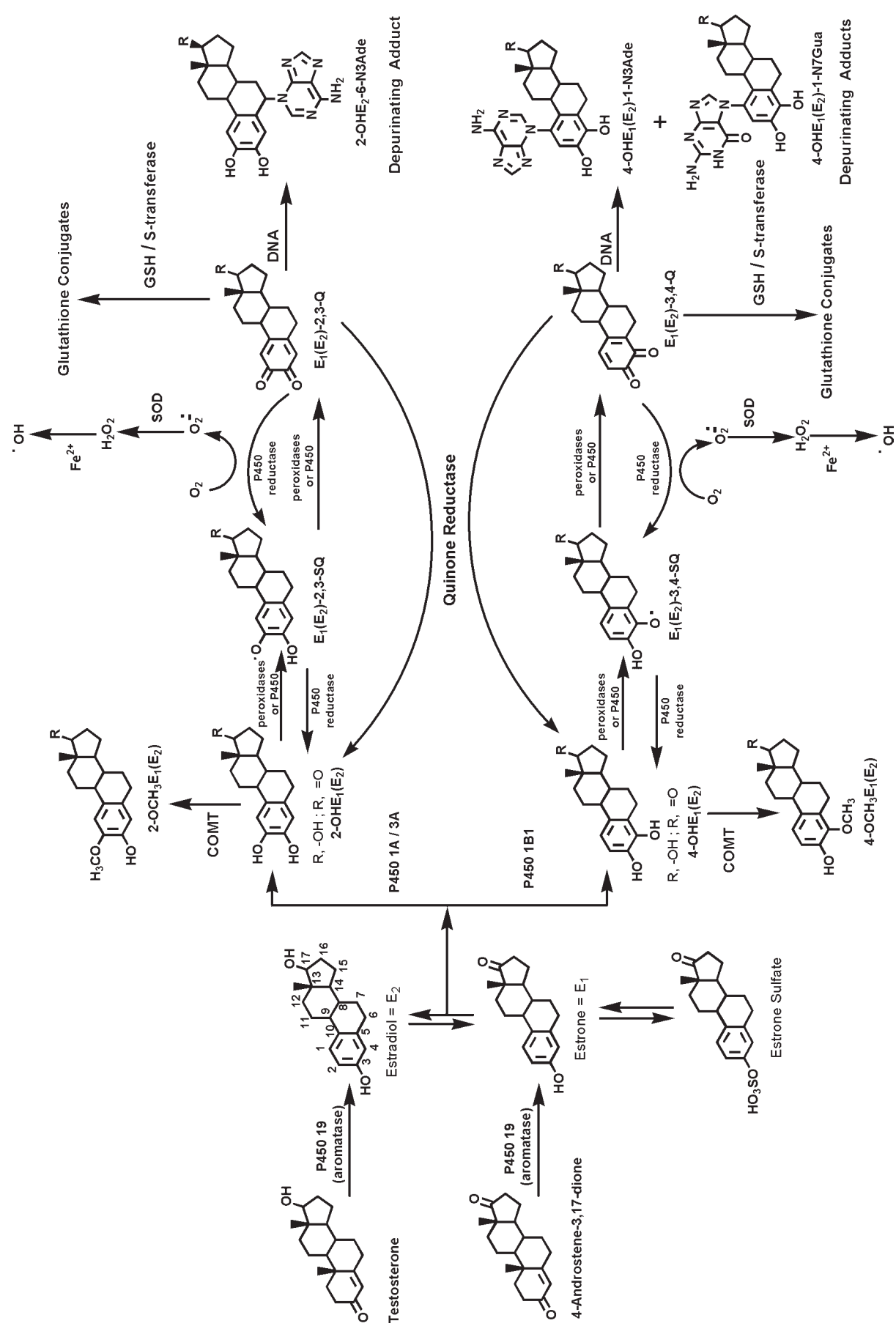

FIGURE 1 – Biosynthesis and metabolic activation of the estrogens, E_1 and E_2. The metabolic activation of E_1 and E_2 leads to 2- and 4-catechol derivatives, which further oxidize to yield the corresponding reactive quinones. The quinones react with DNA to form depurinating DNA adducts. In the deactivation pathway, which operates in parallel, the catechol derivatives are methylated to form methoxy catechol estrogens; in addition, the quinones are reduced by quinone reductase, as well as are conjugated with GSH, and, thus, are rendered harmless. The shift in the apparent balance between these activating and deactivating pathways towards formation of depurinating DNA adducts could lead to the initiation of breast cancer.

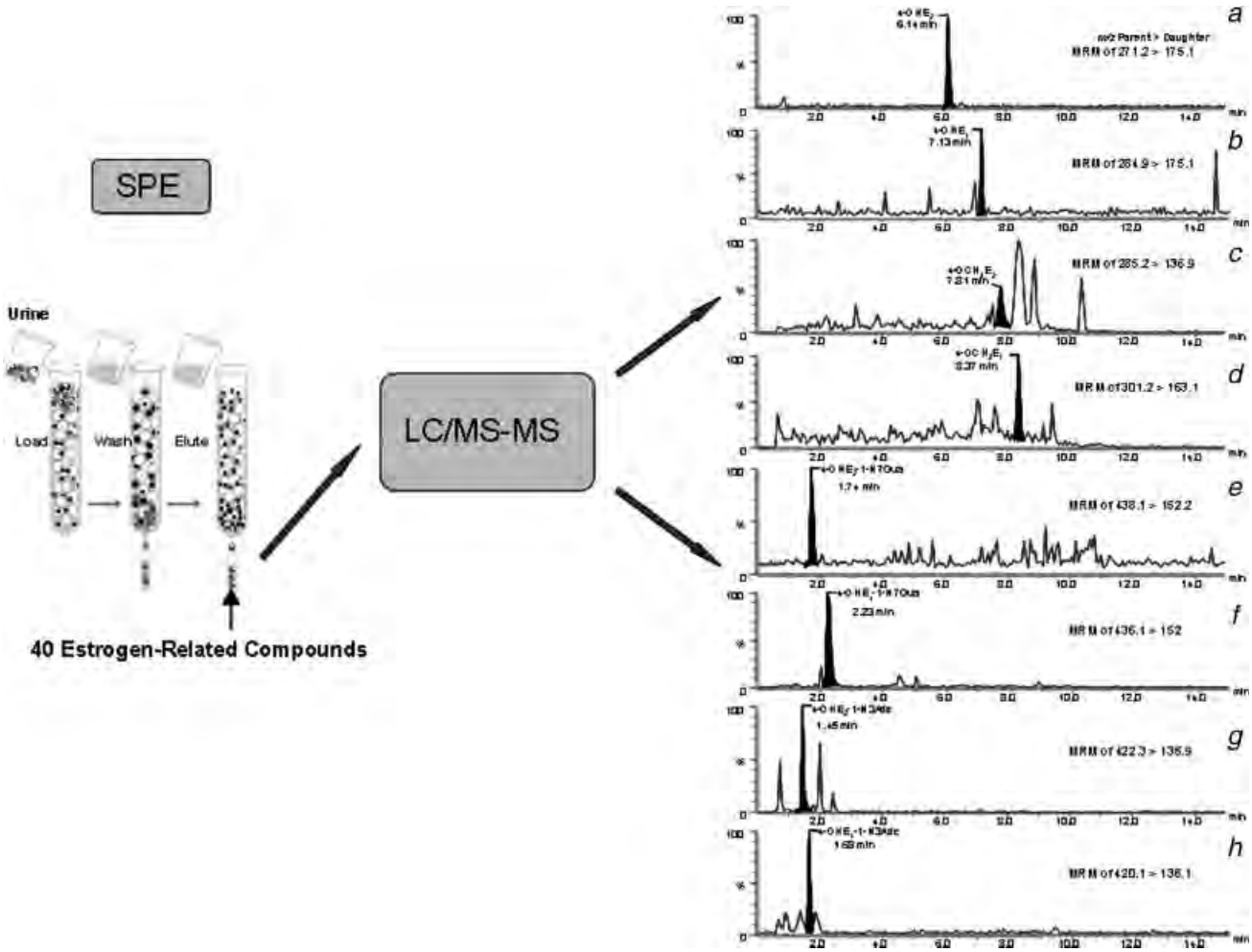

FIGURE 2 – Schematic representation of the steps carried out to purify by SPE and analyze by UPLC/MS-MS the estrogen-related compounds from urine samples. The UPLC/MS-MS chromatograms of (*a*) 4-OHE_2, (*b*) 4-OHE_1, (*c*) 4-OCH_3E_2, (*d*) 4-OCH_3E_1, (*e*) 4-OHE_2-1-N7Gua, (*f*) 4-OHE_1-1-N7Gua, (*g*) 4-OHE_2-1-N3Ade and (*h*) 4-OHE_1-1-N3Ade that are shown in the figure are representatives from the 40 different estrogen-related compounds seen in the urine samples.

on Gail Model score >1.66%, and women with breast carcinoma. The Gail Model takes into account the following factors: age, age at menarche, age at first live birth, number of breast biopsies and history of atypical hyperplasia, number of first degree relatives with breast cancer (mother, sister and daughter) and race. A 5-year Gail Model score of >1.66% is considered high risk.[25]

Material and methods

Materials

Phenyl solid phase extraction (SPE) cartridges were purchased from Varian (Palo Alto, CA). Androstenedione (**1**), (Table I), testosterone (**2**), estrone (E_1) sulfate (**3**), E_2 (**4**), E_1 (**5**), 2-OHE_2 (**6**), 2-OHE_1 (**7**), 16α-OHE_2 (**10**), 16α-OHE_1 (**11**), 2-OCH_3E_2 (**12**), 2-OCH_3E_1 (**13**), 4-OCH_3E_2 (**14**), 4-OCH_3E_1 (**15**), 2-OH-3-OCH_3E_2 (**16**) and 2-OH-3-OCH_3E_1 (**17**) were purchased from Steraloids (Newport, RI). 4-OHE_2 (**8**) and 4-OHE_1 (**9**) were synthesized as previously described.[26] 2-OHE_2-1-SG (**18**), 2-OHE_2-4-SG (**19**), 2-OHE_1-1-SG (**20**), 2-OHE_1-4-SG (**21**), 2-OHE_2-(1+4)-Cys (**22**), 2-OHE_1-1-Cys (**23**), 2-OHE_1-4-Cys (**24**), 2-OHE_2-1-NAcCys (**25**), 2-OHE_2-4-NAcCys (**26**), 2-OHE_1-1-NAcCys (**27**), 2-OHE_1-4-NAcCys (**28**), 4-OHE_2-2-SG (**29**), 4-OHE_1-2-SG (**30**), 4-OHE_2-2-Cys (**31**), 4-OHE_1-2-Cys (**32**), 4-OHE_2-2-NAcCys (**33**) and 4-OHE_1-2-NAcCys (**34**) were synthesized by using the procedure of Cao *et al.*[27] 4-OHE_2-1-N7Gua (**35**), 4-OHE_1-1-N7Gua (**36**), 4-OHE_2-1-N3Ade (**37**), 4-OHE_1-1-N3Ade (**38**), 2-OHE_2-6-N3Ade (**39**) and 2-OHE_1-6-N3Ade (**40**) were synthesized by following the reported methods.[6,7,28] All solvents were HPLC grade and all other chemicals used were of the highest grade available.

Study population

We collected urine from 75 women at 3 different sites: (*i*) at the Center for Mammographic Screening at the University of Naples, Italy (42 women), (*ii*) at the Breast Diagnostic Clinic and Oncology Breast Clinic of the Mayo Clinic, Rochester, MN (18 women) and (*iii*) at the Olson Center for Women's Health, University of Nebraska Medical Center (UNMC), Omaha, NE (15 women). Women were recruited between March 2005 and September 2006 and their ages ranged between 34 and 73 years—healthy women: range, 34–67; mean, 50 ± 8; high-risk women: range, 44–64; mean, 52 ± 6; women with breast cancer: range, 34–73; mean, 54 ± 10.

All women recruited at the University of Naples were healthy (they did not receive a diagnosis of breast cancer at the time of their mammographic test). Among the women recruited at the Mayo Clinic, 12 were classified as high-risk women (Gail Model score = 1.67%–11.7%) and 6 were breast cancer cases. At UNMC, 4 women were healthy, that is, had no known cancer, and 11 were diagnosed with breast cancer. None of the subjects received estrogen-containing treatment for at least 3 months prior

TABLE I – MASS SPECTROMETRIC PARAMETERS[1]

No.	Compound	Mass	ESI mode	Parent (*m/z*)	Daughters (*m/z*)	Cone (volt)	Collision	Retention time	LOD (fmol)
1	Androstenedione	286.2	Positive	287.1	97.1	40	19	8.43	14
2	Testosterone	288.2	Positive	289.2	97.0	40	19	7.97	35
3	E_1-Sulfate	350.1	Negative	249.0	269.0	50	28	6.61	143
4	E_2	272.4	Positive	273.2	135.2	30	14	7.74	184
5	E_1	270.1	Positive	271.2	253.2	25	14	8.43	148
6	2-OHE_2	288.2	Positive	271.2	175.1	30	14	6.74	69
7	2-OHE_1	286.2	Negative	285.0	160.9	65	37	7.3	18
8	4-OHE_2	288.2	Positive	271.2	175.1	30	14	6.14	69
9	4-OHE_1	286.2	Negative	284.9	161.0, 175.1	65	35	7.13	35
10	16α-OHE_2	288.4	Positive	289.0	107.0	25	14	2.42	867
11	16α-OHE_1	286.4	Negative	285.1	145.1	30	15	4.67	349
12	2-OCH_3E_2	302.2	Positive	285.2	136.9, 189.1	32	15	8.25	330
13	2-OCH_3E_1	300.2	Positive	301.2	136.9, 163.1	30	17	8.85	333
14	4-OCH_3E_2	302.2	Positive	285.2	136.9, 189.1	32	15	7.81	66
15	4-OCH_3E_1	300.2	Positive	301.2	163.1, 283.1	30	17	8.37	133
16	2-OH-3-OCH_3E_2	302.4	Positive	285.2	189.1	32	15	8.71	165
17	2-OH-3-OCH_3E_1	300.4	Positive	301.2	163.1	30	17	9.07	33
18	2-OHE_2-1-SG	593.7	Positive	594.1	319.1, 465.0	42	20	1.72	8.4
19	2-OHE_2-4-SG	593.7	Positive	594.0	319.1, 465.4	35	21	2.32	8.4
20	2-OHE_1-1-SG	591.0	Positive	592.1	316.8	45	22	2.65	1.7
21	2-OHE_1-4-SG	591.0	Positive	592.2	317.1, 463.2	40	22	2.65	1.7
22	2-OHE_2-1+4-Cys	407.2	Positive	408.2	319.0	30	17	1.73	12
23	2-OHE_1-1-Cys	405.2	Positive	406.0	316.9	35	15	3.25	6.2
24	2-OHE_1-4-Cys	405.2	Positive	406.2	317.1	30	17	3.25	6.2
25	2-OHE_2-1-NAcCys	449.2	Positive	450.1	162.0, 287.4	25	14	4.07	5.6
26	2-OHE_2-4-NAcCys	449.2	Positive	450.2	162.0, 287.2	30	14	4.07	5.6
27	2-OHE_1-1-NAcCys	447.2	Positive	448.1	162.0, 285.4	30	13	6.05	5.6
28	2-OHE_1-4-NAcCys	447.2	Positive	448.0	162.0, 284.9	35	14	6.05	5.6
29	4-OHE_2-2-SG	593.2	Positive	594.4	318.9, 464.8	42	20	2.33	8.4
30	4-OHE_1-2-SG	591.2	Positive	592.3	317.1, 462.9	45	22	2.65	8.5
31	4-OHE_2-2-Cys	407.2	Positive	408.0	318.9, 286.1	40	16	2.24	2.4
32	4-OHE_1-2-Cys	405.2	Positive	406.0	316.9, 389.0	35	15	2.84	6.2
33	4-OHE_2-2-NAcCys	449.2	Positive	450.1	162.1	35	15	5.91	5.6
34	4-OHE_1-2-NAcCys	447.2	Positive	448.3	161.8	35	14	6.64	2.2
35	4-OHE_2-1-N7Gua	437.2	Positive	438.1	152.2, 272.0	62	38	1.74	2.3
36	4-OHE_1-1-N7Gua	435.2	Positive	436.1	152.0, 271.9	62	38	2.23	2.2
37	4-OHE_2-1-N3Ade	421.2	Positive	422.3	135.9, 257.1	62	45	1.45	5.9
38	4-OHE_1-1-N3Ade	419.2	Positive	420.1	296.0, 136.1	60	44	1.68	2.4
39	2-OHE_2-6-N3Ade	421.1	Positive	422.2	136.0, 287.0	26	10	1.05	1.2
40	2-OHE_1-6-N3Ade	419.1	Positive	420.0	135.9	26	10	1.41	2.4

[1]List of the 40 estrogen-related compounds with the masses of parent and daughter ions and the ionization mode that were used for MRM method optimization. The last column indicates the limit of detection for each compound.

to providing a urine sample. The 3 groups were frequency matched on age, race and menopausal status.

All procedures were approved by the University of Naples, Mayo Clinic and UNMC Institutional Review Boards. Signed consents included authorization to collect and bank urine samples and collect demographic and clinical information.

Sample collection

A standardized method was followed to collect all of the urine samples. A spot urine sample of about 50 ml was collected from each participant and 1 mg/ml ascorbic acid was added to prevent oxidation of the catechol moieties in the various estrogen compounds. The urine samples were aliquoted, frozen and four 10-ml aliquots were transferred to the Eppley Institute, UNMC, on dry ice and were stored at −80°C until analysis. Thus, each analytical sample was thawed only once prior to analysis.

Solid-phase extraction of urine

Two milliliter aliquots of urine were partially purified by SPE. The SPE was performed using a 20-port SPE vacuum manifold with phenyl cartridges (Fig. 2). Urine samples were adjusted to pH 7 with 1 M NaOH or 1 M HCl. For method development and validation, 2-ml aliquots of charcoal-treated human urine samples were spiked with a total of 250, 500 or 1,000 pg of the 40 estrogen-related compounds (final concentration 0.125, 0.25 and 0.50 pg/μl) and loaded onto the phenyl 100-mg cartridges preconditioned with CH_3OH and the loading buffer, 10 mM ammonium formate, pH 7. The cartridges were washed with the loading buffer, and then the compounds of interest were eluted from the cartridge by using an elution buffer, methanol/10 mM ammonium formate, pH 7 (90:10) with 1% acetic acid. This procedure led to enrichment of the 40 estrogen-related compounds after elution. Charcoal-treated urine (2 ml) was used in controls, and the eluates from the SPE cartridges were spiked with 250, 500 or 1,000 pg of the 40 estrogen-related compounds. The eluates from both the experimental and control samples were concentrated using a SpeedVac and lypholizer, and subjected to ultraperformance liquid chromatography/tandem mass spectrometry (UPLC/MS-MS) analysis. To determine the recovery of the standards by the SPE method, comparison was made between the corresponding concentrations of experimental and control samples (Fig. 3). Study samples were cleaned in duplicate by using the above optimized SPE conditions and analyzed by UPLC/MS-MS.

UPLC/MS-MS analysis of urine samples

The 40 analytes (Table I) included the androgens androstenedione and testosterone; the estrogens E_1 sulfate, E_1 and E_2; the catechol estrogens 2-OHE_1(E_2) and 4-OHE_1(E_2); the 16α-OHE_1(E_2); the methylated 2- and 4-catechol estrogens; the 2- and 4-catechol estrogens conjugated with GSH, cysteine (Cys) or *N*-acetylcys-

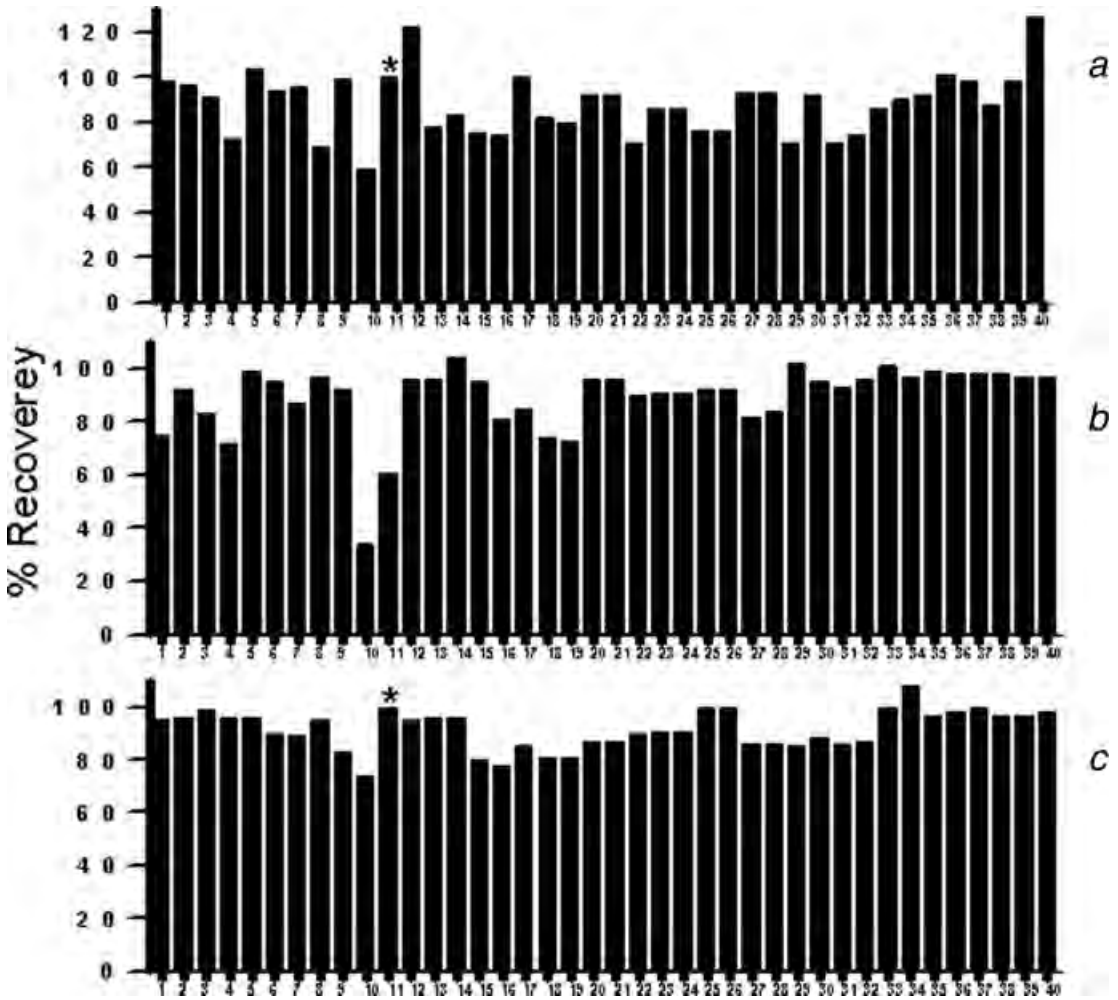

FIGURE 3 – SPE recovery of standard 40 estrogen-related compounds. The 2-ml aliquots of activated charcoal-treated human urine samples were spiked with the total (*a*) 250, (*b*) 500 and (*c*) 1,000 pg of 40 estrogen-related compounds before and after (control) passing over phenyl SPE cartridges. The recovery of each compound was determined by comparing the experimental values to the controls.

teine (NAcCys); and the depurinating DNA adducts of 4-$OHE_1(E_2)$ and 2-$OHE_1(E_2)$. All of the estrogen compounds were analyzed as both E_1 and E_2 derivatives because the interconversion of these 2 estrogens is carried out continuously by 17β-estradiol dehydrogenase.

All experiments were performed on a Waters (Milford, MA) Quattro Micro triple quadrupole mass spectrometer by using electrospray ionization (ESI) in positive ion (PI) and negative ion (NI) mode, with an ESI-MS capillary voltage of 3.0 kV, an extractor cone voltage of 2 V, and a detector voltage of 650 V. Desolvation gas flow was maintained at 600 l/h. Cone gas flow was set at 60 l/h. Desolvation temperature and source temperature were set to 200 and 100°C, respectively. For all the studies, a methanol:-water (1:1) mixture with 0.1% formic acid was used as the carrier solution. ESI interface tuning and mass calibration were accomplished in the PI mode by using a standard sodium iodide-rubidium iodide solution. The test sample (compounds 1 through 40) was introduced to the source at a flow rate of 10 μl/min by using an inbuilt pump. PI or NI detection was used in cases where the sample was readily ionized to cation or anion, respectively. The masses of parent ion and daughter ions were obtained in the MS and MS-MS operations. The parent and daughter ion data obtained for each compound were used to generate the multiple reaction monitoring (MRM) method for UPLC/MS-MS operation (Table I).

Measurements of estrogen-related compounds in urine extracts were conducted by using UPLC/MS-MS. UPLC/MS-MS analyses were carried out with a Waters Acquity UPLC system connected with the high-performance Quattro Micro triple quadrupole mass spectrometer. Analytical separations on the UPLC system were conducted using an Acquity UPLC BEH C18 1.7 μm column (1 × 100 mm) at a flow rate of 0.15 ml/min. The gradient started with 80% A (0.1% formic acid in H_2O) and 20% B (0.1% formic acid in CH_3CN), changed to 79% A over 4 min, followed by a 6-min linear gradient to 45% A, resulting in a total separation time of 10 min. The elutions from the UPLC column were introduced to the Quattro Micro mass spectrometer.

The ionization method used for MS analysis was ESI in both the PI and NI mode. MS-MS was performed in the MRM mode (see above), and resulting data were processed by using QuanLynx software (Waters) to quantify the estrogen metabolites. To calculate limits of detection, various concentrations, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10, 25, 50 and 100 pg/μl, of the analyte were injected to UPLC/MS-MS. The injected amount that resulted in a peak with a height at least 2 or 3 times as high as the baseline noise level was used as the limit of detection (Table I). Pure standards were used to optimize the UPLC/MS conditions prior to analysis. After UPLC analysis, the mean value was calculated for all the compounds obtained from each sample.

Statistical methods

Estrogen-related compounds were compared for control *versus* high risk and for control versus breast cancer using a Mann–Whitney test, with *p*-values adjusted for the 2 multiple comparisons using the Bonferroni method. To account for the multiple hypothesis tests conducted for these variables, a *p*-value <0.01 was interpreted as statistically significant. The log-transformed sum of the ratios of depurinating adducts to the corresponding metabolites and conjugates was compared using a one way ANOVA, and *post hoc* comparisons were made using the method of Bonferroni. Linear regression was used to assess the association between disease status and ratio adjusted for age at recruitment, age at menarche, menopausal status (categorical) and parity for the 56 subjects with patient characteristics available. All the statistics and *p*-values were calculated using SPSS software (SPSS, Chicago, IL).

Results and discussion

Analysis of urine samples

After partial purification of the urine samples by SPE (Fig. 2), we analyzed the 40 estrogen-related compounds using UPLC/MS-MS. The advantage of having MS detector in MRM mode over conventional high pressure liquid chromatography analysis is that number of channels in the detector could be set to specifically and separately identify all the estrogen related compounds (Fig. 2). Each metabolite was detected and identified based on the parameters that are unique to them, such as mass (parent and daughter), retention time and ionization mode (positive and negative) (Table I). The typical spectra of representative estrogen derivatives, which were obtained in a single injection, are shown in Figure 2. The levels of estrogen-related compounds for a high risk woman, measured from single injection, are presented in Table II.

Treatment of urine with glucuronidase/sulfatase led to significant increases (10 to 20-fold) in the levels of E_1 and E_2, while the levels of estrogen metabolites, conjugates and adducts changed marginally and in many cases decreased because of the incubation for 8 hr at 37°C. To avoid artifacts and errors that are introduced by maintaining the urine samples at 37°C for 8 hr, we carried out all the analyses without glucuronidase/sulfatase treatment. Therefore, the observed levels of E_1 and E_2, as reported in Table II, for example, were 10 to 20-fold lower than the total values. Since estrone and estradiol are constantly inter-converting, we have combined estrone and estradiol values of all the derivatives (Tables II and III). The GSH conjugates of estrogen quinones are further converted to Cys and NAcCys conjugates *via* the mercapturic acid biosynthesis pathway.[29] Hence we have combined all the values of 2 conjugates and 4 conjugates (Tables II and III), which reflect the total protection by GSH from 2 or 4 quinones, respectively. The results presented here clearly demonstrate the ability of SPE combined with UPLC/MS-MS analysis to resolve, identify and quantify 40 estrogen-related compounds with accuracy and speed.

The values obtained for the various estrogen-related compounds in 3 groups of women were processed in 2 different ways. First, median values were calculated for all the compounds and their levels were examined in the 3 groups of women (Table III). Then, we used the ratio of depurinating N3Ade and N7Gua adducts to the sum of their respective estrogen metabolites and conjugates in urine samples because the ratio reflects the degree of imbalance in estrogen metabolism that can lead to cancer initiation (Fig. 4). A high ratio of adducts to their respective metabolites and conjugates represents relatively more DNA damage. In contrast, a low ratio of adducts to their respective metabolites and conjugates means that relatively little of the estrogen metabolites reacted with DNA.

Median values of the urinary estrogen-related compounds in the 3 groups of women

Using the newly developed SPE/UPLC/MS-MS methodology, we have analyzed urine samples of various women's groups for estrogen-related compounds. The data obtained were used to calculate median values for each of the 40 compounds (Table III).

The median andostenedione, testosterone, E_2/E_1, 16α-OHE_2/16α-OHE_1, 4-OCH_3E_2/4-OCH_3E_1, 2-$OHE_1(E_2)$ GSH conjugate and derivative values were higher for controls compared to high

TABLE II – REPRESENTATIVE METABOLIC PROFILE OF A URINE SAMPLE OBTAINED FROM A HIGH RISK WOMAN.[1]

No.	Compound	pmole/mg creatinine mean, $n = 2$	Total pmole/mg creatinine
1	Androstenedione	1.56	1.56
2	Testosterone	0.24	0.24
3	E_1 Sulfate	1.81	1.81
4	E_2[4]	5.29	15.93
5	E_1[4]	10.64	
6	2-OHE_2	3.09	3.15
7	2-OHE_1	0.05	
8	4-OHE_2	2.64	2.91
9	4-OHE_1	0.27	
10	16α-OHE_2	12.12	38.64
11	16α-OHE_1	26.52	
12	2-OCH_3E_2	1.95	49.81
13	2-OCH_3E_1	47.87	
14	4-OCH_3E_2	0.41	5.08
15	4-OCH_3E_1	4.67	
16	2-OH-3-OCH_3E_2	1.91	10.27
17	2-OH-3-OCH_3E_1	8.36	
18	2-OHE_2-1-SG	0.17	3.10[5]
19	2-OHE_2-4-SG	0.17	
20	2-OHE_1-1-SG	0.49	
21	2-OHE_1-4-SG	0.47	
22	2-OHE_2-1+4-Cys	0.27	
23	2-OHE_1-1-Cys	0.10	
24	2-OHE_1-4-Cys	0.44	
25	2-OHE_2-1-NAcCys	0.07	
26	2-OHE_2-4-NAcCys	0.07	
27	2-OHE_1-1-NAcCys	0.43	
28	2-OHE_1-4-NAcCys	0.43	
29	4-OHE_2-2-SG	0.51	1.77[6]
30	4-OHE_1-2-SG	0.50	
31	4-OHE_2-2-Cys	0.13	
32	4-OHE_1-2-Cys	0.06	
33	4-OHE_2-2-NAcCys	0.29	
34	4-OHE_1-2-NAcCys	0.28	
35	4-OHE_2-1-N7Gua	0.48	2.81
36	4-OHE_1-1-N7Gua	2.33	
37	4-OHE_2-1-N3Ade	137.78	137.90
38	4-OHE_1-1-N3Ade	0.13	
39	2-OHE_2-6-N3Ade	0.06	0.07
40	2-OHE_1-6-N3Ade	0.02	
(Ratio-4)[2] × 1,000			935
(Ratio-2)[3] × 1,000			1
(Ratio-4) + (Ratio-2) × 1,000			936

[1]Typically, each 2-ml urine sample was analyzed at least 2 times. The data obtained from LC/MS-MS were processed and normalized to creatinine levels. Since the E_1 and E_2 derivatives are interconvertible, the total amount for each E_1 plus E_2 derivative in the various categories are presented in the last column and used for calculating the final ratios of depurinating adducts to the respective metabolites and conjugates.

[2] $$\frac{4-\text{OHE}_1(\text{E}_2)-1-\text{N3Ade}+4-\text{OHE}_1(\text{E}_2)-1-\text{N7Gua}}{4-\text{catechol estrogens}+4-\text{catechol estrogen conjugates}} = \frac{\text{No. 37 + 38 + 35 + 36}}{\text{No. 8 + 9 + 14 + 15 + 29 through 34}}$$

[3] $$\frac{\text{2 - OHE1 (E2) - 6 - N3Ade}}{2-\text{catechol estrogens}+2-\text{catechol estrogen conjugates}} = \frac{\text{No. 39 + 40}}{\text{No. 6 + 7 + 12 + 13 + 16 through 28}}$$

[4]Free E_2 and E_1 in the urine sample.
[5]All 2-$OHE_1(E_2)$ conjugates.
[6]All 4-$OHE_1(E_2)$ conjugates.

TABLE III – URINARY LEVELS OF ESTROGEN COMPOUNDS IN HEALTHY WOMEN, HIGH-RISK WOMEN AND WOMEN WITH BREAST CANCER

No.	Compound	Control (n = 46)		High Risk (n = 12)			Breast Cancer (n = 18)		
		Median	Min–Max	Median	Min–Max	p-value[3]	Median	Min–Max	p-value[4]
1	Androstenedione	9.9	2.1–108	4.2	1.3–11.5	0.003[3]	5.5	0.4–95.1	0.047
2	Testosterone	2.2	0.2–16.5	0.8	0.2–2.8	**0.008**[5]	1.1	0.5–3.7	0.050
3	E_1-Sulfate	5.0	0.1–382	2.4	0.1–10.6	**0.087**	1.1	0.1–121	0.032
4	E_2	31.7	9.1–3865	11.4	4.7–80.0	**0.007**	28.1	3.6–151	0.943
5	E_1								
6	2-OHE_2	10.4	1.7–564	7.3	1.6–26.5	0.035	5.6	0.0–38.8	**0.006**
7	2-OHE_1								
8	4-OHE_2	12.4	2.4–157	8.1	2.9–43.3	0.138	5.2	0.0–28.0	**0.008**
9	4-OHE_1								
10	16α-OHE_2	168	10.3–638	33.7	0.0–279	**0.001**	10.9	0.0–86.3	**<0.001**
11	16α-OHE_1								
12	2-OCH_3E_2	49.7	4.7–568	31.1	6.5–452	0.275	26.8	2.2–171	0.044
13	2-OCH_3E_1								
14	4-OCH_3E_2	73.1	12.6–3979	5.9	1.6–37.1	**<0.001**	21.5	4.5–53.2	**<0.001**
15	4-OCH_3E_1								
16	2-OH-3-OCH_3E_2								
17	2-OH-3-OCH_3E_1								
18	2-OHE_2-1-SG	11.2[1]	0.8–79.8[1]	4.6[1]	1.2–18.7[1]	**0.005**[1]	3.1[1]	1.1–16.9[1]	**0.001**[1]
19	2-OHE_2-4-SG								
20	2-OHE_1-1-SG								
21	2-OHE_1-4-SG								
22	2-OHE_2-1+4-Cys								
23	2-OHE_1-1-Cys								
24	2-OHE_1-4-Cys								
25	2-OHE_2-1-NAcCys								
26	2-OHE_2-4-NAcCys								
27	2-OHE_1-1-NAcCys								
28	2-OHE_1-4-NAcCys								
29	4-OHE_2-2-SG	2.7[2]	0.7–24.6[2]	1.4[2]	0.6–6.8[2]	0.032[2]	1.3[2]	0.4–8.9[2]	0.027[2]
30	4-OHE_1-2-SG								
31	4-OHE_2-2-Cys								
32	4-OHE_1-2-Cys								
33	4-OHE_2-2-NAcCys								
34	4-OHE_1-2-NAcCys								
35	4-OHE_2-1-N7Gua	0.7	0.0–4.8	1.2	0.2–2106	0.238	1.6	0.4–11.8	**0.007**
36	4-OHE_1-1-N7Gua								
37	4-OHE_2-1-N3Ade	0.7	0.0–18.8	1.8	0.5–138	**0.007**	1.2	0.1–288	0.085
38	4-OHE_1-1-N3Ade								
39	2-OHE_2-6-N3Ade	0.1	0.0–6.5	0.1	0.0–0.7	0.999	0.1	0.0–5.4	0.960
40	2-OHE_1-6-N3Ade								

[1]All 2-$OHE_1(E_2)$ conjugates.–[2]All 4-$OHE_1(E_2)$ conjugates.–[3]Bonferroni-adjusted p-value for comparing control *versus* high risk using Mann–Whitney test.–[4]Bonferroni-adjusted p-value for comparing control *versus* breast cancer by using Mann–Whitney test.–[5]Significant p-values are shown in bold.

risk participants, and the median 4-OHE_2-1-N3Ade/4-OHE_1-1-N3Ade values were lower for controls compared to high risk participants. Compared to breast cancer participants, the median 2-OHE_2/2-OHE_1, 4-OHE_2/4-OHE_1, 16α-OHE_2/16α-OHE_1, 4-OCH_3E_2/4-OCH_3E_1, 2-$OHE_1(E_2)$ GSH conjugate and derivative values were higher for controls, while the median 4-OHE_2-1-N7Gua/4-OHE_1-1-N7Gua values were lower for controls. Of particular interest are the significantly lower levels of the methoxycatechol estrogens in the women with breast cancer or at high risk compared to the control women, because this represents a major protective pathway in estrogen metabolism. In addition, the levels of the 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua adducts are higher in the women with breast cancer or at high risk than in the control women, although only 2 of the differences are statistically significant.

Depurinating estrogen-DNA adducts in the 3 groups of women

In the second analysis, the ratios of depurinating N3Ade and N7Gua adducts to the sum of estrogen metabolites and conjugates in urine samples from healthy control women are generally low (Fig. 4). In contrast, high ratios of these adducts to estrogen metabolites and conjugates were observed in urine from high-risk women (Gail Model score >1.66%) and women with breast carcinoma. In general, the value obtained from the high-risk women and women with breast carcinoma derives from the ratio between a high level of adducts and low levels of metabolites and conjugates. In some women, however, the level of adducts was not particularly high, but the levels of metabolites and conjugates were very low, suggesting that a substantial proportion of the metabolites was converted to adducts.

In the sum of the ratios of depurinating adducts to estrogen metabolites and conjugates, the preponderant role is played by the N3Ade and N7Gua adducts of 4-$OHE_1(E_2)$, whereas the adducts of 2-$OHE_1(E_2)$ play a very minor role. For example, for the high-risk subject presented in Table II, the overall adduct ratio is 936, but the contribution of 2-$OHE_1(E_2)$-6-N3Ade is 1, whereas the contribution of 4-$OHE_1(E_2)$-1-N3Ade plus 4-$OHE_1(E_2)$-1-N7Gua is 935. In general, the average contribution of the 2-$OHE_1(E_2)$-6-N3Ade adducts is ~2.5% of the total, whereas the predominant contribution of ~97.5% derives from the 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua adducts. The observation of high levels of depurinating estrogen-DNA adducts in urine from high-risk women, as well as subjects with breast carcinoma (Fig. 4), is consistent with the hypothesis that these adducts are a causative factor in the etiology of breast cancer.

Analysis by subject characteristics

We first analyzed the data using the ratio of depurinating N3Ade and N7Gua adducts to the sum of their respective estrogen

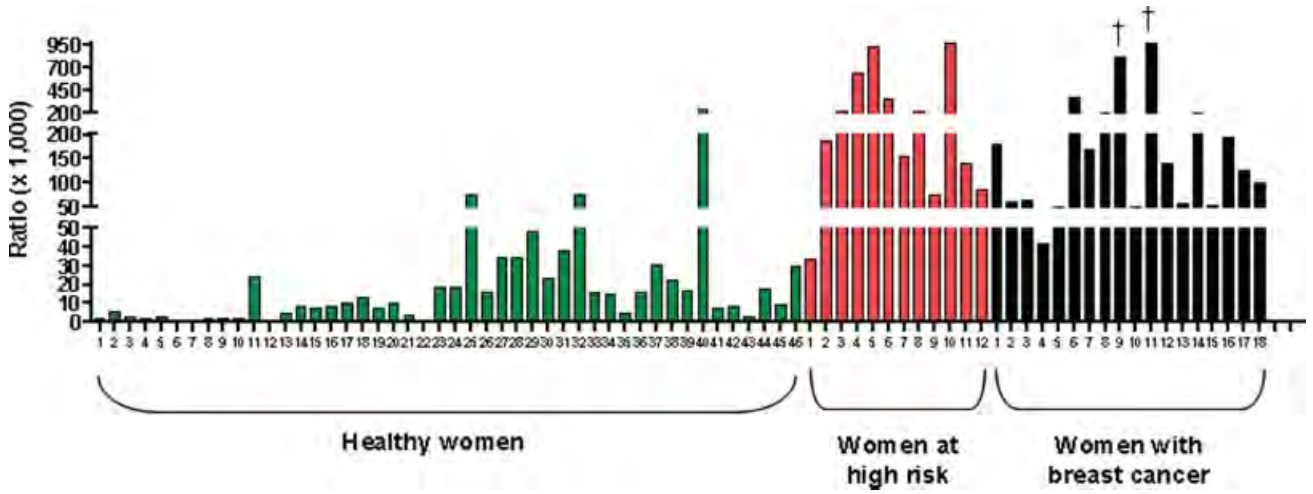

FIGURE 4 – Depurinating estrogen-DNA adducts in the urine of healthy women, high-risk women and women with breast cancer. The ordinate of this bar graph corresponds to the ratio of depurinating DNA adducts divided by their respective estrogen metabolites and conjugates:

$$\left(\frac{4-\mathrm{OHE_1(E_2)}-1-\mathrm{N3Ade}+4-\mathrm{OHE_1(E2)}-1-\mathrm{N7Gua}}{4-\text{catechol estrogens}+4-\text{catechol estrogen conjugates}}+\frac{2-\mathrm{OHE_1(E_2)}-6-\mathrm{N3Ade}}{2-\text{catechol estrogens}+2-\text{catechol estrogen conjugates}}\right)\times 1000$$

The mean sum of the ratios for control women was significantly lower than those for the high-risk women ($p < 0.001$) and women with breast cancer ($p < 0.001$). The mean sums of the ratios for high-risk women and women with breast cancer were not significantly different ($p = 0.62$). †These are 2 urine samples from the same subject, collected 11 weeks apart. Statistical calculations used 1 average value for this subject.

TABLE IV – SUBJECT CHARACTERISTICS

Characteristic	Health status		
	Healthy ($n = 37$)	High risk ($n = 12$)	Breast cancer ($n = 7$)
Age at recruitment in years, mean (SD)	49 (7.85)	52 (6.09)	57 (12.16)
Age at menarche in years, mean (SD)	12 (1.45)	12 (1.44)	13 (1.25)
Menopausal Status, *n* (%)			
Premenopausal	17 (46%)	6 (50%)	3 (43%)
Postmenopausal	20 (54%)	6 (50%)	4 (57%)
Parity			
0	6 (16%)	0 (0%)	0 (0%)
1	3 (8%)	0 (0%)	0 (0%)
2	13 (35%)	7 (58%)	4 (57%)
3	11 (30%)	3 (25%)	0 (0%)
≥4	4 (11%)	2 (17%)	3 (43%)

TABLE V – RESULTS OF UNIVARIATE MULTIVARIATE LINEAR REGRESSION OF RATIO

Covariate	Univariate regression		Multivariate regression	
	Regression coefficient	*p*-value	Regression coefficient	*p*-value
Health status	103.60	0.005	108.56	0.007
Postmenopausal	35.66	0.51	41.18	0.44
Parity	15.01	0.42	−7.29	0.71

metabolites and conjugates in urine samples as a continuous variable. Analysis using one-way ANOVA revealed a significant difference among the groups ($p < 0.001$). Additional *post hoc* analysis using a Bonferroni correction for multiple comparisons revealed significantly higher means for high risk subjects [mean 336.45, standard deviation (SD) 331.92] compared to controls (mean 20.51, SD 37.01, $p < 0.001$) and for breast cancer patients (mean 176.28, SD 205.68, $p < 0.001$). The mean for patients known to be at high risk was not significantly different from that of the breast cancer group ($p = 0.62$).

A limitation of the study is that most of the group of healthy women (42 of 46) were Italian, whereas the remaining healthy women, high-risk women and women with breast cancer were American. All of the subjects in our study, however, were Caucasian. The 3 groups (healthy, high-risk and breast cancer) had similar mean age at recruitment, mean age at menarche and menopausal status (Table IV). These similarities in subject characteristics support the validity of comparing the ratios of adducts to their respective metabolites and conjugates in these 3 groups of women.

Subject characteristics of age at recruitment, age at menarche, menopausal status, and parity were available for 56 of the 75 subjects (Table IV). The mean age of our entirely Caucasian sample was 50 years (SD 8.5). The average age at menarche was 12.0 years (SD 1.4). Only 11% of the women were nulliparous and 43% had at least 2 children. Twenty-six (46%) women were premenopausal at recruitment, 30 (54%) were postmenopausal (they did not have menstrual cycles in the last 12 months before recruitment). Analysis using one way ANOVA revealed that health status, that is breast cancer cases *versus* high risk and healthy individuals, was significantly associated with age at recruitment ($p = 0.048$). Specifically, the mean age (years) at recruitment for healthy women was 49 (SD 7.8), 52 (SD 6.1) for women at high risk and 57 (SD 12.2) for breast cancer cases. Age at menarche was not statistically different across the disease status groups ($p = 0.534$). Analysis using a χ^2 test did not reveal an association between health status and menopausal status ($p = 0.95$) or parity (parous *vs*. nulliparous) ($p = 0.15$).

The correlation coefficient was used to examine the association between the ratio and subject characteristics. We observed evidence of significant correlation between parity and ratio ($r = 0.36$, $p = 0.007$) and marginally significant correlation between the ratio and menopausal status ($r = 0.26$, $p = 0.06$). Age at recruitment and age at menarche were not significantly associated with the ratio.

Linear regression was used to assess the association between disease status and ratio adjusted for age at recruitment, age at menarche, menopausal status (categorical) and parity for the 56

subjects with patient characteristics available (Table V). After accounting for these characteristics, the ratio was significantly associated with health status. Specifically, the multivariate coefficient for disease status (108.6) was statistically significant ($p = 0.007$) in a model that explained 10% ($p = 0.040$) of variance in the ratio after accounting for covariates. All other covariates did not reach the usual level of significance of 0.05 (Table V).

Interpretation of results

The observation of high ratios of depurinating estrogen-DNA adducts to their corresponding metabolites and conjugates in urine samples from both high-risk women and women with breast cancer supports the hypothesis that formation of estrogen-DNA adducts is the first critical step in the initiation of breast cancer.[1] In addition, these results suggest that this assay may provide a diagnostic tool for early detection of breast cancer risk. At this point, we do not know how far in advance this assay would predict the development of a detectable tumor. Further studies are required to address this question.

In addition, we can hypothesize that the ratio of depurinating estrogen-DNA adducts to their metabolites and conjugates can be used to monitor the efficacy of putative preventive compounds in balancing estrogen activation and deactivation. Minimizing formation of catechol estrogen quinones and/or their reaction with DNA should reduce the risk of developing breast cancer.

Conclusions

UPLC/MS-MS can be used to analyze depurinating estrogen-DNA adducts, estrogen metabolites and estrogen conjugates in 2-ml urine specimens. The ratio of adducts to their corresponding metabolites and conjugates provides a biomarker that can be used to distinguish women known to be at high risk of developing breast cancer (Gail Model score >1.66%) and those with breast cancer from healthy control women. The development of such biomarkers could be invaluable in assessing breast cancer risk and response to preventive treatment.

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A-INTRODUCTION

Breast cancer is a malignancy whose dependence on ovarian function was first recognized through the regression of both advanced cancer and metastatic disease induced by oophorectomy in premenopausal women. The correlation of ovarian function with estrogen production, and the isolation of the estrogen receptor protein, combined with the observed greater incidence of estrogen receptor positive tumors in postmenopausal women, led to the identification of a strong association of estrogen dose and length of exposure with increased breast cancer risk. Despite the epidemiological and clinical evidence linking cumulative and sustained exposure to estrogens with increased risk of developing breast cancer (1-4), ***there are, however, three interrelated biological process that needs to be elucidated. First is the understanding of the mechanisms by which estrogen induces cancer, second the cell of origin that is the target of the estrogenic effect and the last one the role of estrogen if any in the epithelial mesenchymal transition in breast cancer.***

Among the mechanisms postulated by which estrogen induce breast cancer, the most widely acknowledged is its binding to its specific nuclear receptor alpha (ER-α) for exerting a potent stimulus on breast cell proliferation through its direct and/or indirect actions on the enhanced production of growth factors. However, the fact that ER-α knockout mice expressing the Wnt-1 oncogene (ERKO/Wnt-1) develop mammary tumors provides direct evidence that estrogens may cause breast cancer through a genotoxic, non-ER-α-mediated mechanism. This postulate is further supported by the observations that when ovariectomized mice are supplemented with E_2 they develop a higher tumor incidence with shorter latency time than controls, even in the presence of the pure antiestrogen ICI-182,780. Experimental studies on estrogen metabolism, formation of DNA adducts, carcinogenicity, mutagenicity, and cell transformation have supported the hypothesis that reaction of specific estrogen metabolites, namely, catechol estrogen-3,4-quinones (CE-3,4-Q) and to a much lesser extent, CE-2,3-Q, can generate critical DNA mutations that initiate breast, prostate and other cancers (5-8).

The second important biological concept is which is the progenitor or target cells or the stem cells in which the estrogen exerts these carcinogenic properties. The content of ER and PgR in the normal breast tissue, as detected immunocytochemically, varies with the degree of lobular development, in a linear relationship with the rate of cell proliferation of the same structures (9). The utilization of a double labeling immunocytochemical technique for staining in the same tissue section of steroid hormone receptors and proliferating cells, i.e. Ki67 positive, has allowed to determine that the expression of the receptors occurs in cells other than the proliferating cells, confirming results reported by other authors. The findings that proliferating cells are different from those that are ER and PgR positive support data that indicate that estrogen controls cell proliferation by an indirect mechanism. ER positive cells treated with antiestrogens secrete TGF-β to inhibit the proliferation of ER negative cells. The proliferative activity and the percentage of ER and PgR positive cells are highest in Lob l in comparison with the various lobular structures composing the normal breast. These findings provide a mechanistic explanation for the higher susceptibility of these structures to be transformed by chemical carcinogens *in vitro*, supporting as well the observations that Lobl are the site of origin of ductal carcinomas. However, the relationship between ER positive and ER negative breast cancers is not clear. We have reported that Lob l contains at least three cell types, ER positive cells that do not proliferate, ER negative cells that are capable of proliferating, and a small proportion of ER positive cells that can proliferate as well. Furthermore, we have observed that when Lob l of normal breast tissue are placed in culture they lose the ER positive cells, indicating that only proliferating cells, that are also ER negative, can survive, and constitute the stem cells. These observations are supported by the fact that MCF-10F, a spontaneously immortalized human breast epithelial cell line derived from breast tissues containing Lob. l and Lob. 2, is ER negative. Recent data on the genomic classification of breast tumors is helping to understand the cell type involved in the emergence of ER positive and ER negative tumors. Breast cancer can be subdivided into five major subtypes based on gene expression profiling: basal-like, Her2(HRBB2)-overexpressing, normal breast tissue-like, and two luminal-like (luminal A and luminal B) subtypes (10). The luminal-like subtypes display moderate to high expression of ERα and luminal cytokeratins, while the basal-like subtype is characterized by ERα(-), ERBB2(-) and high expression of basal cytokeratins 5 and 17. The ERBB2-overexpressing subtype is ERα(-) and is characterized by high

expression of several genes in the ERBB2 amplicon at 17q22.24 (11). ERα(-) tumors are more aggressive than ERα(+) tumors, and the loss of ERα is associated with poor prognosis and poor response to hormonal therapy (12). The outcomes, measured as time to development of distal metastasis, were worst for basal-like and ERBB2-overexpressing, best for luminal A, and intermediate for luminal B subtypes (10, 13, 14). Altogether these data support the concept that estrogen receptors positive and negative may be originated from two different cell populations as postulated earlier (9).

The third biological problem inherent to breast cancer is that their progression toward malignancy is accompanied by loss of epithelial dedifferentiation and a shift towards a mesenchymal phenotype. This process has been referred to as epithelial to mesenchymal transition or EMT, that exacerbates motility and invasiveness of many cell types and is often considered a prerequisite for tumor infiltration and metastasis.

In order to definitively outline the pathways through which estrogens act as carcinogens in the human breast either through the receptor pathway or through a genotoxic effect in a specific cell type of the breast and how these early events are associated with specific EMT phenotype it is needed an experimental system in which E_2 by itself or its metabolites induce transformation of human breast epithelial cells (HBEC) in a well controlled environment, preferentially *in vitro.* Towards this purpose we have developed an *in vitro*/*in vivo* system in which the carcinogenic action of estrogen on human breast epithelial cells (HBECs) is demonstrated, *in vitro*, by estrogen-mediated transformation of the spontaneously immortalized HBEC line, MCF-10F. Treatment of these cells with either 17β-estradiol (E_2) or its DNA reactive catechol metabolites results in acquisition of transformed phenotypes including colony formation in agar methocel, decreased ductulogenesis, and increased invasiveness (6). Of great interest, MCF-10F cells do not have detectable levels of ERα, and the *in vitro* cell transformation is not abrogated by co-treatment of these cells with antiestrogen ICI-182-780, supporting a non ERα-mediated mechanism (16). Recently, complete neoplastic transformation of MCF-10F cells has been demonstrated by the formation of tumors in an appropriate heterologous host. MCF-10F cells transformed with 70 nM E2 and then selected by Matrigel invasion chambers form tumors when injected into the mammary fat pad of the abdominal region of severe combined immune depressed (SCID) mice. Cell lines established from these tumors also form tumors in SCID mice. The tumors were poorly differentiated adenocarcinomas characteristic of primary breast tumor (15) that are ER (-), PgR(-) and ERB2 (-), mimicking one the basal cell type described earlier(11).

Furthermore, as a model of estrogen-mediated malignant transformation of HBECs, this is a unique system for identifying the temporal acquisition of changes in genome structure and gene expression that correspond to the transformed phenotype culminating in tumorigenesis. For this purpose, we performed Affymtrix 100k single nucleotide polymorphism (SNP) arrays to measure chromosomal copy number (CN) and loss of heretozygosity (LOH), and HG-U133_Plus_2 array to measure mRNA expression. By integrating these data we were able to identify associations between CN changes, LOH and tumorigenic phenotype, as well as the related changes in transcript expression. Furthermore, the functional influences of these changes on the biological processes and canonical pathways were elaborated by combining the gene expression profiles with their functional annotations in various knowledge bases using sophisticated algorithms and software. Progressive changes in both genome structure and gene expression profiles were observed, and these changes may be responsible for the malignant cell transformation. The up- and down-regulated genes were enriched in the regions of CN amplification and deletion, respectively. The breast cancer stem cell markers CD44 and CD24 (17) displayed $CD44H^{high}/CD44E^{-}/CD24^{-}$ pattern in the tumorigenic cells in current study. These changes will be described by Dr Thomas Sutter in section IV. Moreover, these cells demonstrated a phenotype of epithelial-mesenchymal transition (EMT). Functional analysis identified several deregulated pathways, including integring signaling, glutathione metabolism and apoptosis. Together, these analyses revealed new aspects of the cell biology of E_2-mediated malignant cell transformation in ERα(-) HBECs.

B-BODY

B-i- Methods and procedures.

B-i-a- *Ductulogenic assay.*

MCF-10F, E2 70 and C5-T8 (9) were suspended at a final concentration of 7.5 X 10^3 cells/ml in 89.3% (Vitrogen100) collagen matrix (Collagen Co., Palo Alto. CA, USA) and plated into 24-well chambers pre-coated with 89.3% of collagen base. They were fed with fresh high calcium media. The cells were examined under an inverted microscope for seven days. At the end of observation period the structures were photographed, fixed in 70% alcohol solution and processed for histological examination.

B-i-b-Histological and immunohistochemical analyses

Tissues fixed in alcohol 70%, dehydrated ad embedded in paraffin were cut at 5 μm thickness and stained with hematoxylin and eosin for histological analysis. For immunohistochemical analysis, tissue sections were mounted on aminoalkylsilane-coated or positively charged slides, deparaffinazed, rehydrated and incubated in 2% hydrogen peroxide at room temperature for 15 minutes for quenching endogenous peroxidase activity. The sections were sequentially incubated in two changes of Target Retrieval Solution at 98°C for 5 minutes each. All tissue sections were incubated in diluted normal blocking serum for 20 minutes. Excess serum was blotted from the slides and sections were incubated with the following antibodies: HHF35 a mouse anti-human muscle actin primary antibody, epithelial membrane antigen (EMA) clone E29, AE1, anti-human low molecular weight cytokeratin (Biogenex, San Ramon,CA), E-Cadherin, (Becton Dickinson Biosciences), Vimentin monoclonal mouse anti-human antibody (Dako Cytomation Colorado Inc.), and Fibronectin P1H11 mouse monoclonal raised against a cell binding domain of fibronectin of human origin (Santa Cruz Biotechnology, Inc., CA). After incubation in a humidity chamber at 4°C overnight, sections were washed in buffer and incubated with horse biotinylated secondary antibody (Vector Laboratories. Inc., Burlingame, CA, USA) at room temperature for 30 minutes followed by a 30 minutes incubation with Vectastain Elite avidin-biotin complex kit (Vector Laboratories), washed in PBS buffer, and incubated in peroxidase substrate solution containing hydrogen peroxide and 3, 3' – diaminobenzidine-HCL for 2 minutes. Sections incubated with no immune serum were used as negative controls. All sections were lightly counterstained with hematoxylin. . Immunostaining was evaluated by examination of slides under a bright field microscope, and graded according to the intensity of the brown staining.

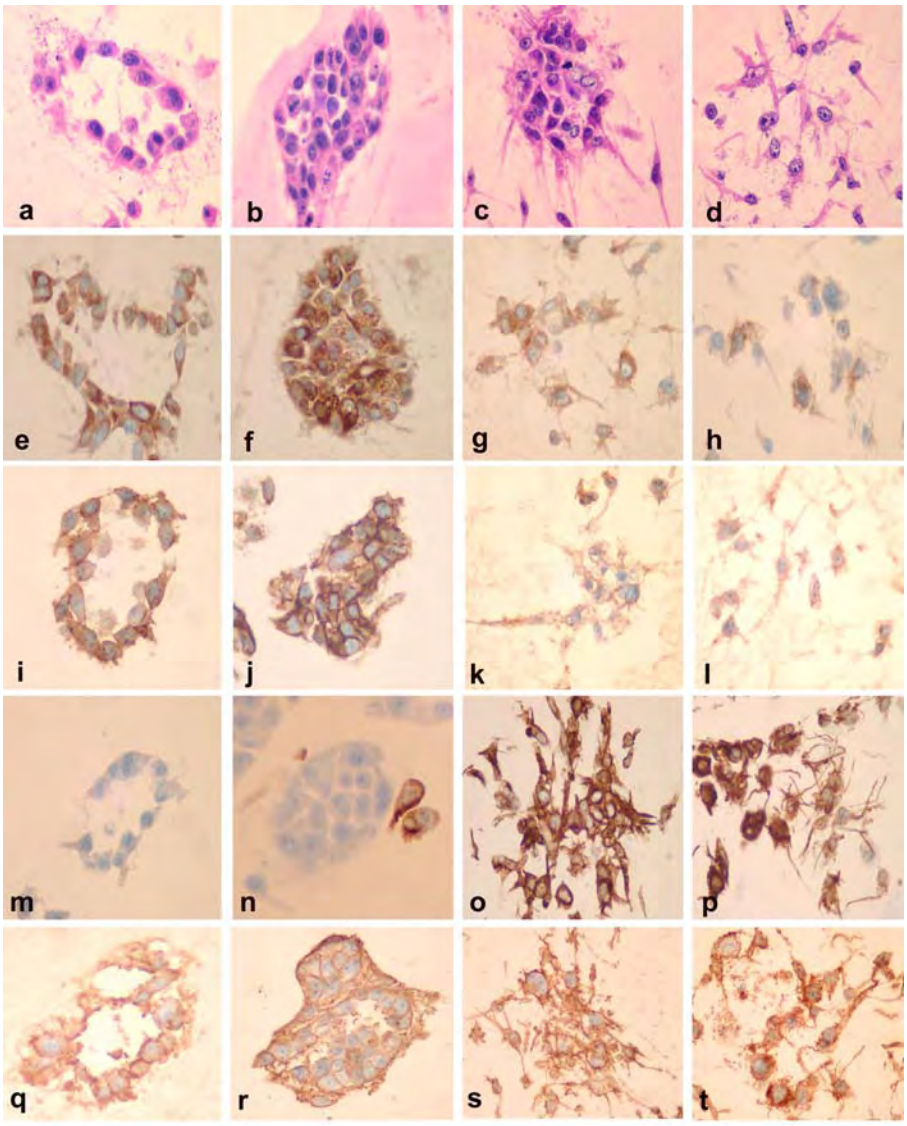
B-i-c-Real time RT-PCR.

Total RNA was isolated from growing cells at 70% - 80% confluence using Trizol (Life Technologies, Inc.) according to manufacture's instructions. The RNA was treated with DNase I (Invitrogen) and cleaned using RNeasy kit (Quiagen). The concentration and quality of RNA were determined spectrophotometrically and by capillary gel eletrophoresis (Agilent 2100 Bioanalyser, Palo Alto, CA). Real time reverse transcriptase PCR (Real time RT-PCR) was used to quantify the expression of E-cadherin, TGFß1, TGFß2, h-RAS, TWIST1, SNAIL2, SMAD5, FN1, CEACAM1 and JAG1. The TaqMan One Step RT-PCR kit (Applied Biosystems) was used and the assays ran using Applied Biosystems 7900 HT instrument. The Ct (threshold cycle) was calculated using three 25ng of RNA sample for each cell line. The TATA box-binding protein (TBP) was used as endogenous RNA control and each sample was normalized on the basis of its TBP content. The Ct was defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe pass a fixed threshold above baseline. The SDS 2.1 software based on the comparative Ct method was used for data analysis. The comparative method calculates the relative gene expression using the following equation: relative quantity= $2^{-\Delta\Delta Ct}$ (User Bulletin 2, Applied Biosystems). For each gene, the expression level was compared to expression in the parental cell line MCF-10F.

B-ii- Results.

B-ii- a- ***Ductulogenic assay.***

MCF-10F, E2 70 and C5-T8 cell lines presented with different phenotypes when growing in collagen matrix. MCF-10F exhibits a duct-like growth pattern (Figure 1a) whereas E2 70 cell line forms spherical masses that in the histological sections resemble a ductal hyperplasia or carcinoma in situ (Figure 1b). The cell line C5-T8 grows in an invasive spread pattern with no structure formation (Figure 1c) or as single spindle cells resembling an invasive ductal carcinoma (Figure 1d).



In order to further characterize the cell phenotype we studied the expression of muscle actin protein using HHF35 primary antibody and the expression of epithelial membrane antigen using EMA Mc-5 primary antibody (Table 1). There was no HHF35 staining in MCF-10F, E2 70 and C5-T8 cells (Table 1) indicating that they are not myoepithelial cells, otherwise, all cell lines were positive for EMA (Figure 1 e-h) revealing their breast. epithelial nature. Keratin was significantly reduced from the MCF10F to the C5-T8 cells (Table 1). E cadherin was strongly positive in MCF10F cells and start decreasing the reactivity in the E2 70 cells (Figures 1i and 1j), for being almost negligible in C5-T8 cells (Figures 1k and 1l). Analyzing vimentin expression, MCF-10F and E2 70 cell lines are negative (Figures 1m and 1n), whereas the C5-T8 cells have a strong dark brown staining (Figures 1o and 1p). The staining for fibronectin also shows an increase in the intensity for
the C5-T8 (Table 1).

Figure 1: a: Histological section of MCF10F cells growing in collagen matrix, H&E x40; b:E2 70 cells growing in collagen matrix, H&E x40;c and d: C5-T8 cells growing in collagen matrix, H&E x40;; e, i, m and q:MCF10F cells reacted with EMA, E-Cadherin, Vimentin, and Fibronectin respectively (40X); f, j, n and r; E2 70 transformed cells reacted EMA, E-cadherin, Vimentin and fibronectin respectively (40X); g, h, k, l, o, p and s t: C5-T8 cells reacted with EMA, E-cadherin, Vimentin and fibronectin respectively (40X);

Table 1. Immunohistochemical expression profile of human breast epithelial cells transformed with estradiol

Antibody	MCF-10F cells	E2 70 cells	C5-T8 cells
EMA	++++	++++	++
HHF35	-	-	-
AE1 cytokeratin	++++	++	-
E-cadherin	++++	++	-
Vimentin	+*	-	++++
Fibronectin	+++	++	++++

Negative (-), weak (+), moderate (++), marked (+++) and strong (++++).

B-ii-b-Gene expression study.

We have determined by RT-PCR the expression of E-cadherin, TGFß1, TGFß2, h-RAS, TWIST1, SNAIL2, SMAD5, FN1, CEACAM1 and JAG1 genes (Figure 2). We observed a reduction in E-cadherin expression in E2 70 cells and a completely lost in C5-A8-T8 cells. TGFß1, TGFß2, CEACAM1 and JAG1 were down regulated in E2 70 and C5-A8-T8 cells. SMAD5 and h-RAS were up regulated in the tumorigenic C5-A8-T8 cells whereas FN1, TWIST1 and SNAIL2 were up regulated in C5-A8-T8 and down regulated in E2 70. Figure 2 shows the relative expression of EMT related genes during MCF-10F cell transformation.

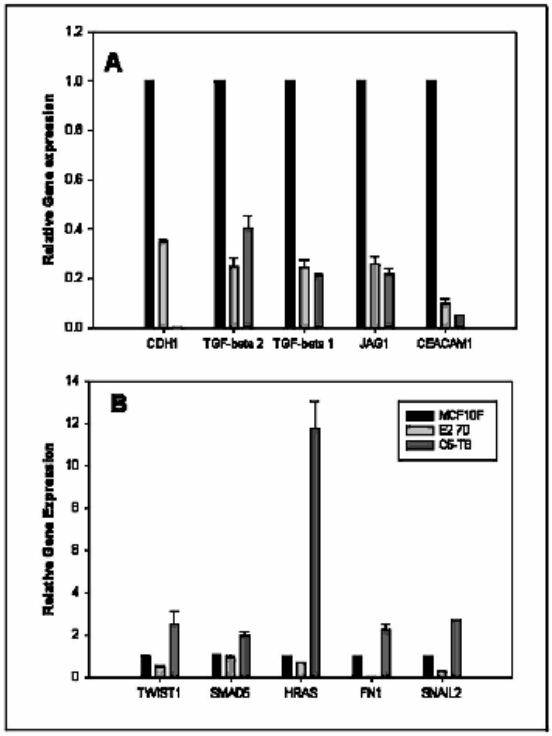


Figure 2: Comparative expression of genes related to EMT process during the neoplastic transformation of breast epithelial cells by estrogen.

B-iii next proposed Plan of research

To publish all the information gathered during this grant award period.

C-KEY RESEARCH ACCOMPLISHMENTS

1- Treatment of MCF-10F cells with 17β-estradiol results in malignant cell transformation. In this model, cells from the initially 17 beta estradiol transformed foci are nontumorigenic, whereas cells selected from this population by migration through Matrigel invasion chambers form solid tumors in SCID mice. Integrated analysis of genotyping and gene expression arrays revealed progressive genomic changes. Chromosomal amplifications were found in 1p36.12-pter, 5q21.1-qter and 13q21.31-qter; losses were detected in 8p11.21-23.1 and all of chromosome 4. In cell line established from tumors, additional losses were found in 3p12.1-14.1, 9p22.1-pter and 18q11.21-qter. Functional profiling of dysregulated genes revealed inhibition of apoptosis and glutathione metabolism pathway, epithelial-mesenchymal transition and $CD44H^{high}/CD44E^{-}/CD24^{-}$ phenotype in tumorigenic cells, and deregulation of integrin signaling pathway in both nontumorigenic and tumorigenic cells.

2. We have demonstrated that Epithelial-mesenchymal transition (EMT) in epithelial cells is an important component of 17 beta estradiol induced neoplastic transformation. We have evaluated the expression of different genes related to EMT such as E-cadherin, TGFß1, TGFß2, h-

RAS, TWIST1, SNAIL2, SMAD5, FN1, CEACAM1 and JAG1 using the *in vitro- in vivo* model of the estrogen induced cell transformation developed in our laboratory. The E2-transformed MCF-10F (E2 70) cells and the tumorigenic cell line C5-A8-T8 (C5-T8) exhibit progressive loss of ductulogenesis as demonstrated by growth in collagen matrix. MCF-10F cells form ductal structures while E2 70 cells form solid spherical masses that in histological sections exhibit a pattern of growth resembling ductal hyperplasia or carcinoma *in situ.* The tumorigenic cells C5-T8 did not form structures on collagen acquiring an invasive pattern with spindle like features. We have observed a reduction in E-cadherin expression in E2 70 cells and a completely lost in C5-T8 cells. TGFß1, TGFß2, CEACAM1 and JAG1 were down regulated in E2 70 and C5-T8 cells. SMAD5 and h-RAS were up regulated in the tumorigenic C5-T8 cells whereas FN1, Twist1 and Snail2 were up regulated in C5-T8 and down regulated in E2 70. We conclude that the lost of expression of TGFß1, TGFß2, CEACAM1 and JAG1 are related to ductulogenesis and branching and the overexpression of h-RAS with loss of E-cadherin expression and up modulation of TWIST1, SNAIL2 and SMAD5 expressions are involved in the EMT modulation.

D-REPORTABLE OUTCOMES

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E-CONCLUSIONS

During the fourth year of our award we have clearly demonstrated for the first time, that 17β-estradiol induces complete *in vitro* transformation of human breast epithelial cells, as evidenced by the expression of anchorage independent growth, loss of ductulogenesis in collagen, invasiveness in Matrigel, and tumorigenesis in SCID mice. Functional profiling of dysregulated genes revealed inhibition of apoptosis and glutathione metabolism pathway, epithelial-mesenchymal transition and $CD44H^{high}/CD44E^{-}/CD24^{-}$ phenotype in tumorigenic cells, and deregulation of integrin signaling pathway in both nontumorigenic and tumorigenic cells. We also have concluded that the lost of expression of TGFß1, TGFß2, CEACAM1 and JAG1 are related to ductulogenesis and branching and the overexpression of h-RAS with loss of E-cadherin expression and up modulation of TWIST1, SNAIL2 and SMAD5 expressions are involved in the EMT modulation.

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SPECIFIC AIM 3 - GUTTENPLAN

A. Introduction

Epidemiological studies have implicated steroidal estrogens in the etiology of breast cancer [1, 2]. Stimulation of proliferation in ER positive cells has been hypothesized to increase the probability that some endogenous damage to DNA will be converted to mutations before it is repaired [1, 3]. However, it has also been proposed that estrogen metabolites themselves may be genotoxic and give rise to DNA damage and mutations [3-5]. Metabolism of estradiol to the catechol estrogens, 2-OHE_2 and 4-OHE_2, followed by oxidation to E_2-2,3 or E_2-3,4-semiquinone and then E_2-2,3 or E_2-3,4-quinone (Fig. 1) has been proposed to lead to mutagenesis by redox cycling between the semiquinone and quinone forms, with the concomitant production of ROS [3-5]. It has also been shown that the E_2-3,4-quinone reacts with DNA in vitro leading predominantly to the depurinating adducts, 4-OHE_2-1-N3adenine and 4-OHE_2-N7guanine [5]. E_2-2,3-quinone also gives rise to a depurinating adduct, 2-OHE_2-6-N3adenine and stable guanine and adenine adducts[1] [5]. Under equivalent conditions, E_2-3,4-quinone forms much higher levels of depurinating adducts than E_2-2,3 quinone [5]. 4-OHE_2 and 2-OHE_2 have been detected in breast tissue from women with breast carcinoma, but the former compound was present in much higher levels and was also present in higher levels than in breast tissue from women without breast cancer [6, 7]. To further support the genotoxic hypothesis, it is important to demonstrate that 4-OHE_2 is mutagenic and, as a corollary, the mutagenic activity should be greater than that of 2-OHE_2.

Early studies on genotoxicity of estrogens and their metabolites failed to detect any mutagenic activity (reviewed in [3]), perhaps because of inappropriate concentrations of estrogens or inadequate test systems or conditions [3]. More recent studies in cultured cells have reported that the 4-OHE_2 precursor, E_2, is weakly mutagenic in V-79 cells in the HGPRT assay [8]. The dose-response curve was unusual in that mutagenesis decreased at higher doses [8]. A small number of mutants were sequenced, and mutations at A:T base pairs were most common, but a number of mutations were identical, suggesting they resulted from clonal expansion rather than independent mutations. Small deletions were also observed with similar frequencies. Other studies have reported that E_2, 4-OHE_2, and 2-OHE_2 all led to strand breaks as detected in the comet assay in MCF-7 cells, but differences in potencies between these compounds were not apparent [8]. However, cells were assayed immediately after treatments, so that accurate repair of at least some of the DNA damage would likely not have had time to occur. It has also been reported that E_2, and 4-OHE_2, transforms cells [9, 10].

Certain in vivo studies also demonstrate that estradiol or its metabolites are carcinogenic or mutagenic in rodents. E_2-3,4-quinone induces mutations in the H-ras gene of SENCAR mouse skin [11]. 4-OHE_2 is also carcinogenic in Syrian Golden hamsters and CD-1 mice [3] whereas the 2-OHE_2 is only marginally carcinogenic at best [3]. An important link in the evidence for a genotoxic mechanism contributing to carcinogenicity of estrogens would be a demonstration that catechol estrogens are mutagenic. We have measured the mutagenic activities of 4-OHE_2 and 2-OHE_2 in lacI rats and a lacI rat embryonic cell line, BB® rat2, and determined the mutational spectrum of 4-OHE_2.

[1] Zahid, et al., Chem. Res. Tox., submitted

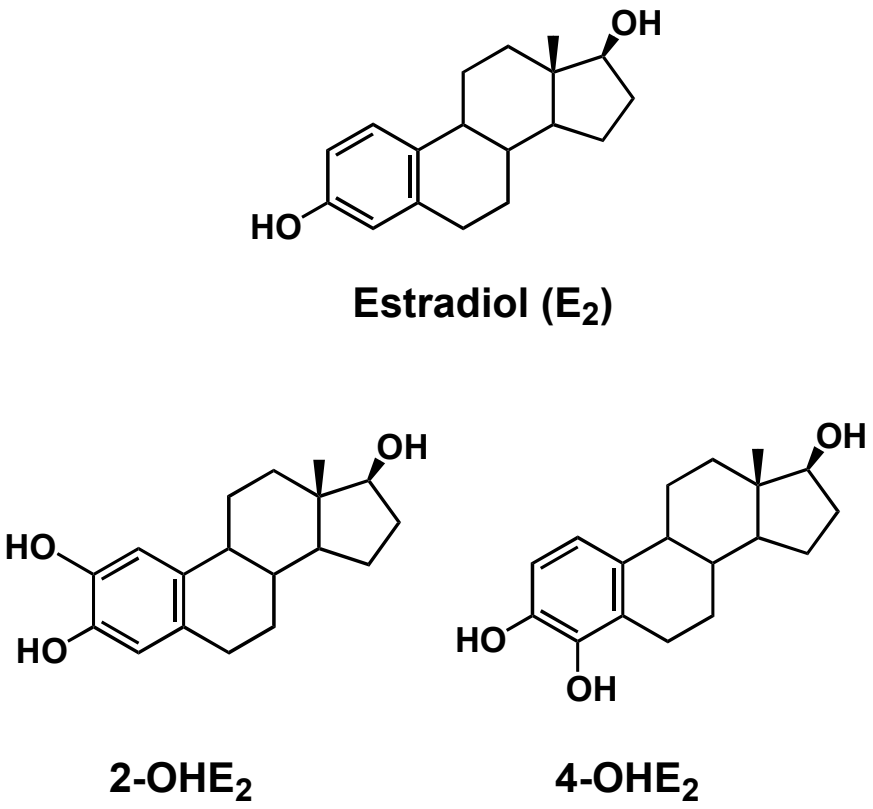

Figure 1 Structures of estradiol and hydroxylated metabolites.

B. Body

Methods

Treatment conditions
Studies in cells.

Cells were grown in 10 cm^2 petri dishes in phenol red-free Dulbecco's Modified Eagle Medium (Invitrogen Corp., Grand Island, NY) containing 10% charcoal/dextran-treated fetal calf serum (Hyclone, Logan UT) to a density of about 10^6 cells/dish. Glutamine, G418-sulfate and penicillin-streptomycin (Mediatech, Inc., Hemdom, VA) were added to concentrations of 0.29 mg/ml, 50 i.u., 50 ug/ml and 0.22 mg/ml, resp. When treated with catechol estrogens or solvent alone, the medium was changed to phenol red-free DMEM without serum, and after each treatment, the medium was removed, cells were rinsed with PBS, and the phenol red-free DMEM containing charcoal-treated fetal calf serum was added. All treatments were carried out on two or three replicate plates and at the doses shown in Figs. 2 and 3, the entire experiment was repeated. Values in figures represent the average MF from each separate dose in the two separate experiments. When treated multiple times, treatments were for 18 hr, every other day, except weekends. After three treatments, cells were trypsinized one portion was used for a mutagenesis assay and the rest was replated at a density of 2 x 10^6 cells/dish for subsequent treatment. The lineage of each plate was retained after plating. After 6 treatments the cells were

grown to about 80% confluence and harvested. The doubling time was about 24 hr in DMEM containing serum.

An initial dose-response experiment for single treatments was carried out to determine a dose range with minimal toxicity. Cells from each treatment group were counted after three treatments and surviving cell numbers/plate were, within experimental error, the same for all doses except the highest dose tested (400 nM for 4-OHE_2 and 800 nM for 2-OHE_2) where there were slightly fewer cells (ca. 80% cell survival). For single treatments, doses above 400nM for 4-OHE_2 and 1360 nM for 2-OHE_2 resulted in toxicity of > 50%.

Studies in LacI rats

Groups of 6-8 Fisher 344 *lacI* rats were implanted with E_2, 4-OHE_2, 2-OHE_2, and 4-OHE_2 + E_2 in silastic tubing and euthanized 20 weeks later. DNA was isolated from 4 upper inguinal mammary fat pads from each rat. Mutagenesis in each DNA was analyzed separately, and the 4 individual mutant fractions (MF's) averaged to obtain the MF for each rat. The treatments were carried out twice for E_2, 4-OHE_2 and the control, with similar results; and the combined results along with those for other treatment groups is given in the table below.

Mutagenesis

After treatment of the cells or rats, DNA was extracted using a Recoverase kit (Stratagene, LaJolla, CA) as per manufacturer's instructions, which involves isolation of nuclei, cell lysis, digestion with protease K and RNAse and dialysis on a membrane. Phage packaging was carried out using a phage packaging mix prepared from bacterial strains E coli NM759 and BHB2688 generously supplied by Dr. Peter Glazer (Yale, Univ. School of Medicine, New Haven, CT) according to published methods [12]. The cII mutagenesis assay was then employed.

The BB® rat2 cell line contains a lambda shuttle vector that includes the bacterial lacI locus and also the cII gene, which is the target for the mutagenesis studies. It also obviates the potential for ex vivo mutations that could complicate results. This assay detects mutations at the cII locus and possibly the regulator cI locus [13] [14-17]. The cII protein is a positive regulator of gene transcription that controls the decision between lytic or lysogenic development pathways in phage-infected cells. In appropriate E. coli (E. coli 1250) host cells, under specified conditions (25° C) only mutants give rise to phage plaques, whereas at 37° C all infected cells give rise to plaques, providing a phage titer [13] [14-17]. The ratio of mutant to non-mutant plaques is the measure of mutagenesis, the mutant fraction (MF). Each DNA sample was assayed at least twice and at least 15 mutants were accumulated for each DNA sample.

Amplification and sequencing

Mutants were cored from petri dishes and the agar plug was mixed with 100 ul phage buffer. 10 ul of the buffer was then spread on a selective plate to confirm mutant phenotype and purify mutant phages. Both control and 4-OHE_2- induced mutant plaques were randomly selected from selective plates containing packaged DNA samples isolated from several plates/dose. The purified mutant plaques were the subjected to amplification and sequencing of the cII gene by PCR. Sequencing was performed by Roderick Haesevoets, University of Victoria, B.C., Canada.

Amplification: Primer sequences, forward: 5'-AAAAAGGGCATCAAATTAAACC-3', reverse: 5'-CCGAAGTTGAGTATTTTTGCTGT-3'
Reaction Mixture (100.0 μL reaction) H_2O 59.1 μL, 100mM dNTP mix,1.0 μL10X buffer,10.0 μL cII forward primer (10.0 μM), 2.0 μL cII reverse primer (10.0 μM) 2.0 μL, 50mM $MgCl_2$3.5 μL, Taq, 2.4 μL, sample, 20.0 μL.10X buffer:100 mM Tris HCl pH 9.0, 500 mM KCl, 1.0% TritonX100.
PCR conditions: 94.0^o, 4.0 min; 30 cycles: 95.0^o, 30 sec; 55.0^o; 30 sec; 72.0^o, 2.5 min; 4.0^o, hold.
Purification: PCR Product Pre-Sequencing Kit (USB); as per kit instructions.
Sequencing: Primer sequences,cII forward: 5'-ACCACACCTATGGTGTATG-3',
cII reverse, 5'-GTCATAATGACTCCTGTTGA-3' (only used to confirm a mutation if the sequence from cII forward primer is not clear)
Reaction Mixture: CEQ Dye Terminator Cycle Sequencing (DTCS) Quick Start Kit (Beckman-Coulter).
PCR conditions: 96.0^o, 5.0 min, 30 cycles: 96.0^o, 20 sec; 52.0^o, 25 sec; 60.0^o, 4.0 min;
4.0^o, hold
Electrophoresis/Base Calling/Trace Generation: Sequencing was done on a CEQ8000 Capillary Electrophoresis DNA Sequencer (Beckman-Coulter).
Analysis software: SeqMan II v6.1 (DNASTAR).

Statistics

Differences in MF's between the 4-OHE_2 -treated and control groups were analyzed for significance using a two-tailed Student's *t*-test. At least four measurements (2 experiments and duplicate measurements for each dose) were used for each point.

Results

Studies in cells

Mutant fractions:

Initial experiments conducted at doses from 10 - 6800 nM 4-OHE_2 failed to detect any significant increase in mutant fraction. Since previous studies had indicated that 4-OHE_2 was not mutagenic, and its precursor, E_2, was more mutagenic at certain lower doses than higher doses, we tested multiple low dose exposures to 4-OHE_2. 4-OHE_2 induced a dose-dependent increase in mutant fraction from 50 - 200 nM (Fig. 2). This was marginally apparent at three treatments and clearer after six treatments. In both cases the 200 nM treatments resulted in statistically significant increases over controls. Also, the mutant fraction declined at 400 nM after both three and six treatments.

Using similar protocols, for single and multiple treatments, it was not possible to detect any induction of mutagenesis over background by 2-OHE_2 (Fig.3). 2-OHE_2 was about 3X less toxic than 4-OHE_2 and was less effective in cell transformation assays [10] therefore the doses used were about 3X greater than those used for 4-OHE_2.

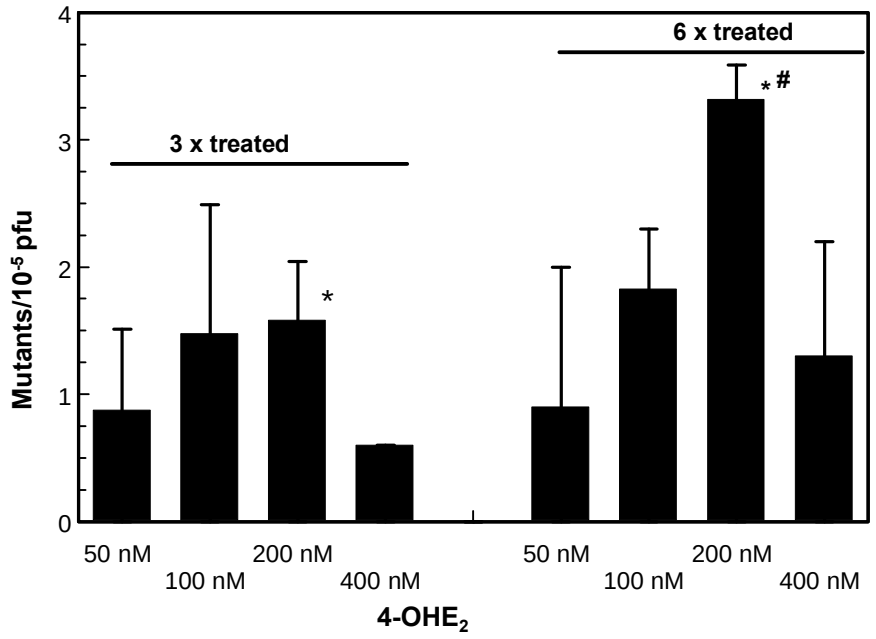

Figure 2. Effects of dose on the mutant fraction in the cII gene from 4-OHE_2 -treated BB® rat2 cells. In order to normalize results from different experiments, background mutant fractions (from solvent controls) have been subtracted from all values. The background obtained in each individual experiment was subtracted from the corresponding mutant fraction of the 4-OHE_2-treated group. Backgrounds ranged from 2.2 - 3.5 mutants/10^5 pfu. Asterisk indicates $P < 0.05$vs. solvent controls; number sign indicates $P < 0.05$ in corresponding 3x-treated vs. 6x-treated.

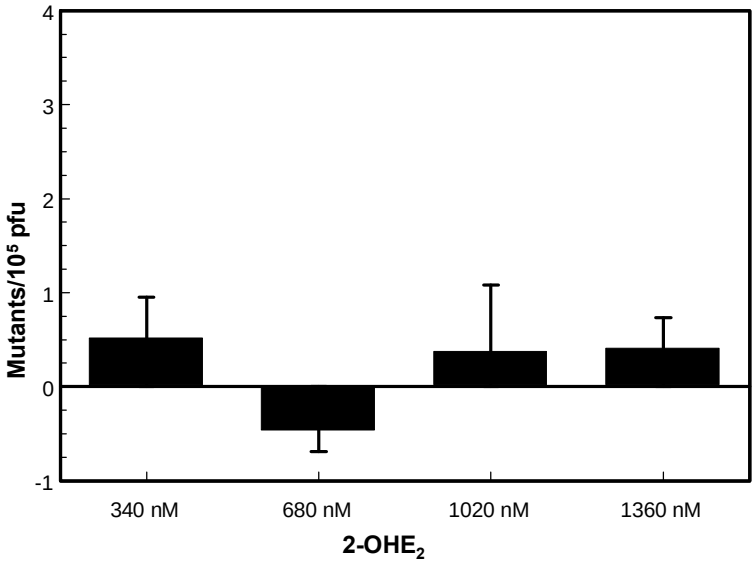

Figure 3. Effects of dose on the mutant fraction in the cII gene from 2-OHE_2 -treated BB® rat2 cells. Cells were treated six times with the above doses.
The mutant fraction from background (solvent alone) plates was 4.3 +/- 0.5 pfu and has been subtracted from the above values.
Error bars, standard errors.

Mutational spectra

The mutational spectra from the 4-OHE_2 treated and control plates were compared (Tables 1-3). A major apparent difference between the two groups was the higher percentage of mutations at A:T base pairs in the mutants from the 4-OHE_2 -treated cells than in controls (*ca.* 24% vs. 6%). Also, there was a higher fraction of GC:AT transitions in controls and a higher fraction of these were at CpG sites. In addition, the fraction of GC>TA mutations was higher in the 4-OHE_2 -treated group than the controls. A number of the mutations in both 4-OHE_2 and control cells (25% and 34% resp.) were identical. For the control cells, it was assumed these all resulted from clonal expansion and redundant mutants were only counted once for each experiment. Two experiments with 2-3 plates/dose were conducted and each experiment utilized a freshly thawed culture of BB® cells. For the 4-OHE_2-treated cells it was assumed that identical mutants from different plates were independent, since they might have been induced by the 4-OHE_2 separately on each plate, but those identical mutants on the same plate presumably resulted from clonal expansion and were only counted once. The clonal mutants have not been included in tables 1 and 2.

Table 1: DNA sequence context of mutations among mutants in the 6 times 4-OHE_2-treated BB® rat2 cells[1,2,3,4]

position	change	context
-14	g>a	cta agg aaa
-13	g>t	cta agg aaa
1	A>G	cat ATG GTT
2	T>C	cat ATG GTT
3	G>A	cat ATG GTT
24	C>G	CGC AAC GAG
24	C>G	CGC AAC GAG
24	C>G	CGC AAC GAG
29	C>T	GAG GCT CTA
32	T>C	GCT CTA CGA
42	G>C	ATC GAG AGT
57	C>A	CTT AAC AAA
58	A>G	AAC AAA ATC
65	C>T	ATC GCA ATG
73	G>C	CTT GGA ACT
86	C>A	AAG ACA GCG
118	A>G	CAG ATC AGC
119	T>G	CAG ATC AGC
129	G>T	AGG TGG AAG
148	A>C	CCA AAG TTC
163	C>A	CTG CTT GCT
169	G>C	GCT GTT CTT
179	G>C	GAA TGG GGG
191	A>G	GTT GAC GAC
196	G>A	GAC GAC ATG
205	C>G	GCT CGA TTG
241	A del. (6>5)	ATT AAA AAA
241	A del. (6>5)	AAT AAA AAA
241	A del. (6>5)	AAT AAA AAA
241	A del. (6>5)	AAT AAA AAA
190-192	GAC del. (3>2)	GTT GAC GAC
29-30	CT del. (2>1)	GAG GCT CTA
btwn. 178-179	G insert. (6>7)	GAA TGG GGG
btwn. 178-179	G insert. (6>7)	GAA TGG GGG

1. Altered nucleotides are underlined.
2. Mutants taken from plates treated 6x with 100 and 200 nM 4-OHE_2
3. Eleven redundant mutations not included in the table
4. Position no. 1 corresponds to the start of the coding sequence, and upper case letters refer to sequences in the coding region of the cII gene.

Table 2. DNA sequence context of mutations among mutants in 6 times solvent-treated BB® rat2 cells[1,2,3]

position	change	context
24	GAC del. (3>2)	GTT GAC GAC
34	C>T	CTA CGA ATC
52	C>T	TTG CTT AAC
64	G>A	ATC GCA ATG
73	C>G	CGC AAC GAG
88	G del. (6>5)	GAA TGG GGG
113	C>T	AAG TCG CAG
113	C>A	AAG TCG CAG
118	A>G	CAG ATC AGC
179	G del. (6>5)	GAA TGG GGG
179	G>C	CTT GGA ACT
196	G>A	GAC GAC ATG
241	A del. (6>5)	ATT AAA AAA
248	G>C	AAA CGC CCG
190-192	G insert. (6>7)	GAA TGG GGG
btwn. 178-179	G insert. (6>7)	GAA TGG GGG
btwn. 178-179	C>G	CGC AAC GAG

1. Altered nucleotides are underlined.
2. Nine redundant mutations not included in the table.

A summary table is included below.

Table 3. Mutational spectrum of 4-OHE_2 induced and spontaneous mutations: numbers and percentages of mutants

Mutation class		**4-OHE_2**		**control**	
		number	**%**	**number**	**%**
Total		34	100	17	10
Base substitutions		26	76.5	12	70.6
Transitions		10	29.4	7	41.2
	GC:AT	5	14.7	6	35.3
	at CpG sites	1	2.9	4	23.5
	AT:GC	5	14.7	1	5.9
Transversions		16	47.1	5	29.4
	GC:TA	5	14.7	1	5.9
	GC:CG	8	23.5	4	23.5
	AT:CG	3	8.8	0	0
	AT:TA	0	0	0	0
Deletions		5	14.7	4	23.5
	1 bp	3	8.8	3	17.7
	2 or 3 bp	2	2.8	1	5.9
Insertions (-1bp)		3	8.8	1	5.9

Studies in rats

Mutant fractions:

Two experiments were carried out because of the realtively small changes in mutant fractions. In each the rats were treated for 20 weeks. Statistically significant differences in the MF's were observed between the control and the E_2, 4-OHE_2, 4-OHE_2 + E_2 groups (Table 4). The highest mutant fractions was observed for the combination of 4-OHE_2 and E_2. 2-OHE_2 was not mutagenic. We did not observe a dose response for mutagenesis induced by 4-OHE_2. Additionally we investigated whether the estrogenic compounds, diethylstibesterol (DES) or 4-OH-diethylstilbesterol were mutagenic in rats. At 5 mg/rat the MF's for DES and 4-OH-DES were 1.55 +/- 0.39 and 1.43 +/- 0.48 resp.

Table 4. Effects of E_2, 4-OHE_2 and 2-OHE_2 on the mutant fraction in the cII gene from 4-OHE_2 -treated BB® lacI Fisher rats

treatment	Dose (mg)	MF (mutants/ 10^5 pfu)	SD
2-OHE_2	5	0.96	0.25
4-OHE_2	2.5	1.65	0.69 *
4-OHE_2	5	1.48	0.38 *
E_2	5	1.43	0.49
4-OHE_2 +E_2	2.5 + 2.5	1.93	0.50 *
control		1.05	0.41

*, $p < 0.05$ vs. control in 2-tailed t-test

The data for the individual rats is included below

Table 5. Mutant fractions in individual rats treated with E_2, 4-OHE_2 and 2-OHE_2

	Experiment 1				
rat #	**control**	**4-OHE2**	**E2**	**4-OHE2**	**2-OHE2**
1	0.63	1.90	1.18	0.80	
2	0.71	2.15	1.62	2.30	
3	0.90	2.30	2.07	2.15	
4	1.17	2.13	2.43	1.30	
5	1.85	1.78	2.88	0.71	
6	0.55	1.43	2.66	1.90	
				2.43	
	Experiment 2				
7	1.12	1.07	1.42		0.94
8	0.74	1.67	0.98		1.00
9	0.85	1.29	1.09		0.55
10	0.77	1.09	1.37		0.97
11	1.47	1.91	0.74		1.34
12	1.34	1.18	2.33		0.78

13	0.00	1.97	2.19		1.13
14	1.17	1.44			
15	1.64				
16	1.33				

Mutational spectra in rats

The mutational profiles for E_2, 4-OHE_2, 4-OHE_2 + E_2 and control mutations were analyzed. A major difference was that the fraction of mutations at AT base pairs was 19% in the 4-OHE_2 groups, 21% in the 4-OHE_2 + E_2 group, but only 10 and 11% in the E_2 and control groups resp. (Figure 4). This is consistent with ability of 4-OHE_2 to form depurinating adducts with adenine. A table presenting the raw data follows:

Table 6 . Distribution of mutations induced by E_2, 4-OHE_2, E_2 + 4-OHE_2 and in control rat mammary tissue in vivo

	numbers of mutants							
	GC AT	**GC TA**	**GC CG**	**AT GC**	**AT CG**	**AT TA**	**DEL/INS**	**total**
4-OHE2	63	20	4	9	3	5	12	116
E2 + 4-OHE2	17	3	5	3	2	5	3	38
E2	16	10	3	1	0	2	8	40
control	37	17	6	7	3	1	16	87

The results are also depicted graphically in Fig. 4 in terms of mutant fractions at GC and AT base pairs.

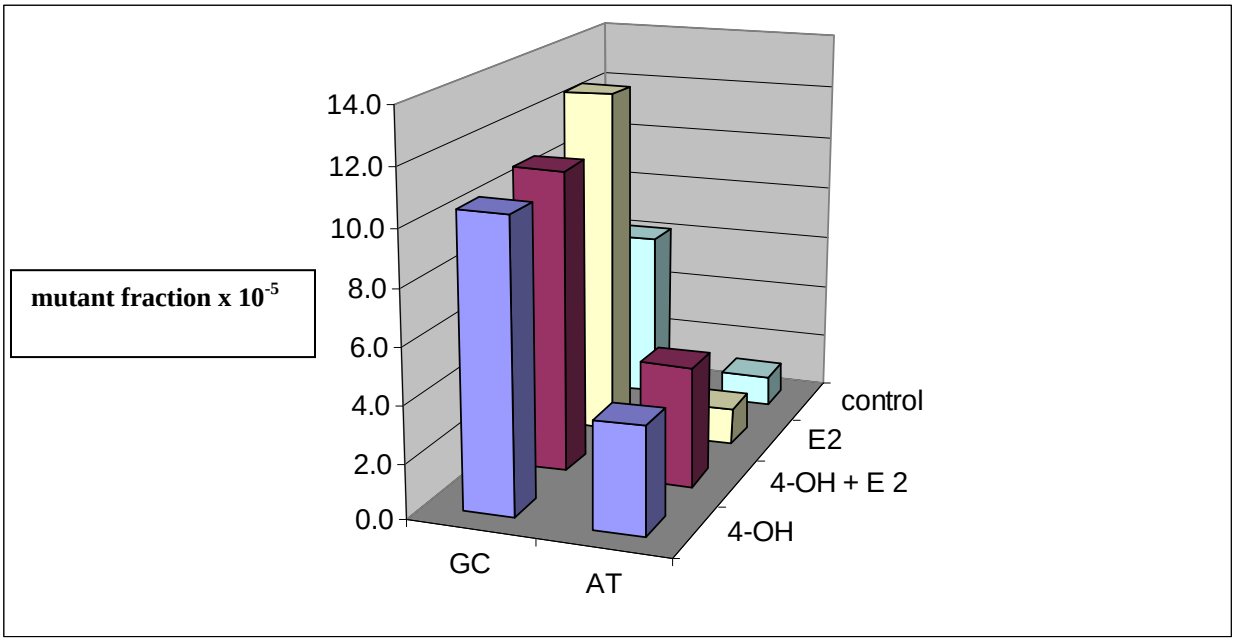

Figure 4. Mutant factions induced at AT and GC base pairs by E_2, 4-OHE_2, E_2 + 4-OHE_2 and in control rat mammary tissue in vivo.

Next proposed plan of research

To finish analyzing data and publish results

C. Key research accomplishments

A major goal of this project was to demonstrate that estradiol and its 4-hydroxymetabolite were mutagenic and that mutagenesis results from a genotoxic mechanism. We have demonstrated that 4-OHE_2 is mutagenic *in vitro* and *in vivo*. Although it could be argued that mutagenicity results from increased cell turnover, we have also observed that there is an increase in mutations at AT base pairs relative to controls, and this provides evidence that DNA modifications occur.

D. Reportable results

1. Zhao Z. Kosinska W. Khmelnitsky M. Cavalieri EL. Rogan EG. Chakravarti D. Sacks PG. Guttenplan JB. Mutagenic activity of 4-hydroxyestradiol, but not 2-hydroxyestradiol, in BB rat2 embryonic cells, and the mutational spectrum of 4-hydroxyestradiol. Chemical Research in Toxicology. 19(3):475-9, 2006.
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4. Khmelnitsky, M., Kosinska, W., Cavalieri, E.C., Rogan, E.G., Chakravarti, D., Guttenplan, J.B. Mutagenic activity and specificity of estradiol and metabolites in lacI rat mammary gland and liver. Proc Am Assoc Cancer Res 2007
5. Khmelnitsky, M., Kosinska, W., Cavalieri, E.C., Rogan, E.G., Chakravarti, D., Guttenplan, J.B. Mutagenic activity and specificity of estradiol and metabolites in lacI rat mammary gland and liver (manuscript in preparation).

E. Conclusions: In both *in vitro* and *in vivo* systems the 4-OHE_2 led to a modest increase in mutagenesis (ca. 50%). However, as estrogens are present in many tissues and particularly high in premenopausal women, it is not unexpected that they would be weak mutagens. If this were not so, they would lead to high incidences of cancer. As the pathways leading to metabolism of estrogens to genotoxic compounds has been elucidated, this knowledge provides leads for intervention and prevention of estrogen-induced breast cancer.

F. References

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Progress Report Specific Aim #4

Richard J. Santen MD.

A. Introduction:

Breast cancer occurs in approximately 240,000 women in the United States annually with 50,000 expected deaths (1)). Improved diagnostic and treatment strategies have decreased mortality by 25 % over the past two decades (2,3), but the physical and psychological burdens of surgery, radiotherapy, hormonal therapy and chemotherapy are substantial. Accordingly, breast cancer prevention represents a major focus of current research. (4). Rational strategies for breast cancer prevention require understanding of the molecular mechanisms of carcinogenesis. Genetic factors predominate in a substantial fraction of women with breast cancer and include *known mutations* of *BRCA 1* and *2, CHEK2, TP53, LKB-1*, and *PTEN* in 5-10% (5-7) and *unknown mutations*, as determined by identical twin studies, in another 10-15% (6). Hormonal factors can modulate the development of breast cancer in known mutation carriers as exemplified by the 50% reduction in lifetime incidence by early oophorectomy in *BRCA 1* and *2* carriers (8,9). Hormonal factors also exert major modulating effects on breast cancer incidence in women without a demonstrable genetic component (10). Epidemiologic and experimental data implicate estradiol (E_2) as a key hormone in breast cancer development.

The precise molecular mechanisms whereby E_2 influences breast cancer development are not well understood. Current theories suggest that two separate pathways potentially responsible: one ER dependent and the other, ER independent and mediated by genotoxic metabolites of estradiol (11). While mechanistically distinct, these pathways could act in a synergistic, additive, or independent fashion. Current concepts suggest that ER dependent mechanisms involve increased stimulation of cell proliferation with mutational errors during replication (12,13). ER independent genotoxic metabolites could produce mutations through damage to DNA (14-17). Yager and Davidson recently outlined the four oxidative steps potentially involved (11): **(1)** 16-hydroxylation with covalent binding of 16-hydroxy-E_2 to proteins and consequent DNA damage; **(2)** redox cycling with formation of oxygen free radicals resulting from reversible inter-conversions between the catechol-estrogens and estrogen-quinones; **(3)** 2-hydroxylation with formation of stable DNA adducts resulting from further oxidation to their respective quinones; and **(4)** 4-hydroxylation with formation of unstable, depurinating estrogen-quinone adducts. Data published within the past year by collaborators in Specific Aims #1-3 provide strong support that the 4-hydroxylation pathway can exert mutagenic as well as carcinogenic effects.

The rationale for current preventative strategies with anti-estrogens rests primarily upon the receptor dependent pathway of carcinogenesis (4,18). However, the possibility that E_2 can contribute to the development of breast cancer via ER independent mechanisms is of key clinical importance (11). Newer agents being considered for breast cancer prevention, the aromatase inhibitors, block E_2 synthesis and abrogate both ER dependent and independent pathways whereas the antiestrogens only block the ER (19-21). Provided that genotoxic pathways do contribute to breast cancer development, the

aromatase inhibitors should be superior to the anti-estrogens for the prevention of breast cancer.

Specific Aim #4 was designed to provide proof of the principle that E_2 can influence breast carcinogenesis through an ER independent mechanism in an animal model. Because of the strong genetic components of breast cancer in women, we chose an experimental model carrying a breast cancer oncogene in which to examine the modulatory effects of E_2. This model involved transgenic mice engineered to over-express the Wnt-1 oncogene in the mammary gland which experience a 100% incidence of breast cancer (22). To dissect out the separate roles of ER independent from ER dependent pathways, we cross bred these animals into an ERα knock-out (ERKO) background. E_2, administered to castrate ERKO/Wnt-1 animals, caused a dose response related induction of breast tumors, providing strong evidence of an ER independent effect. To ensure complete blockade of any residual ER function, we administered the selective ER down-regulator fulvestrant to ERKO/Wnt-1 mice and demonstrated that E_2 still resulted in enhanced tumor formation. Our data provide evidence that ER independent as well as ER dependent mechanisms of E_2 carcinogenesis are operative in this animal model and provide a rationale for use of aromatase inhibitors rather than anti-estrogens for prevention of breast cancer in women.

B. Methods and Procedures:

Animals. Wnt-1 transgenic animals were originally obtained from Dr. Harold Varmus and bred at the National Institute of Environmental Health Sciences, Research Triangle Park (22-24). These animals were then cross bred with ERα +/- heterozygous mice to generate Wnt-1 transgenic mice in the presence of ERα. Breeding pairs were then sent to the University of Virginia to establish a colony on site. The mice were housed and treated in accordance with the NIH guide to Humane Use of Animals in Research. All surgical procedures were approved by the Animal Care and Use Committee at the University of Virginia. Genotyping was performed using tail DNA and PCR as previously described (22). Studies providing full characterization of the phenotypic, biologic, and biochemical properties of these animals have been recently reviewed (22,25,26).

Preparation of whole mounts. The whole mounts from excised mammary glands were fixed and stained as previously described (27).

"Estradiol Clamp" method. Silastic tubes of 0.19 cm internal diameter were filled with estradiol/cholesterol mixtures at various ratios. The lengths of the filled part of Silastic tubes were 2.5, 5 or 7.5 mm, respectively. Our prior studies validated the ability to clamp plasma E_2 levels ranging from 20 to 800 pg/ml over a two-month period (28). As further evidence of the validity of this technique, we also demonstrated linear dose responses in uterine weight in castrate mice (29). In the present study, the levels of plasma E_2 were clamped at 5, 10, 80, and 240 pg/ml. The implants used contained the following estradiol/cholesterol ratios and lengths: 1:39/2.5 mm (5 pg/ml); 1:19/2.5 mm (10 pg/ml); 1:3/2.5 mm (80 pg/ml) and 1:3/7.5mm (240 pg/ml). These implants were inserted under the skin in the back of mice and changed every two months. Fulvestrant was administered by subcutaneous injection once per week at a dosage of 5 mg/mouse.

Endogenous and exogenous gene expression assays. The ERE-TATA-luciferase reporter system was previously described in detail (30). Progesterone receptors A and B

were detected on western blots using the monoclonal antibody against the progesterone receptor (Cell Signaling Technology, Beverly, MA) (30).

Measurements of Depurinated Estrogen-adenine conjugate

CE/FASS. The analysis of extracts obtained from tissue samples was performed using the GPA100 capillary electrophoresis system (Groton Biosystems). The UV/VIS detector model Lambda 1010 (Bischoff Chromatography) was used for detection of absorption based electropherograms measured at 214 nm. Capillary electrophoresis (CE) with field amplified sample stacking (CE/FASS) using a reversed polarity mode was used for detection. UV transparent capillaries (80 cm x 74 μm ID and 366 μm OD) were obtained from Polymicro Technologies (48 cm effective length to the detection window were used). Prior to use the capillary was conditioned for 30 minutes with 0.1M sodium hydroxide, water, and running buffer. The pH of the running buffer (Tris 25 mM plus 0.5% SDS) was adjusted to pH = 3.2 using phosphoric acid. The final extract obtained from the 0.5 g of tissue using a solid phase extraction (SPE) and incubated with helix palmitase (sulfatase/beta-glucuronidase enzyme) to release free steroids was dissolved in 100 μL of methanol/water (1:1) mixture and used for subsequent analysis. Typically, about 2 μL of this extract was diluted by a factor of 10 and used in CE analysis. The water plug was injected first into the capillary (p = 35 mba, t = 0.3 min) followed by the sample (p = 35 mba, t = 0.6 min). The applied potential for separation was –5 kV (and/or -3 kV) and typical separation was performed at T = 28^{o}C.

Biochip. IgG 15G8-4H12 monoclonal antibodies (MAb) and 4-OHE$_2$-1- N3Ade-SAMSA conjugates were developed specifically for the detection of estrogen-derived DNA adducts. MAbs were purified through protein G column. PS-10 ProteinChip from Ciphergen Biosystems were used for analysis. The pre-activated chip surface had carbonyl diimidazole (CDI) activated amine surface, which can covalently bind MAb through an amine group. First, each spot of the biochip (with a diameter of ~3 mm) was wetted with 2 μL of 50% acetonitrile/water solution for about 1 min. After removing acetonitrile/water solution, about 2 μL PBS was added to the active surface and incubated in a humidity box for 1 min at room temperature. After removing PBS buffer (following procedures established by Ciphergen Biosystems), about 2 μL of PBS buffer was added again to the active area of the chip along with 3 μL of 15G8-4H12 MAb (c = 2.4 mg/mL). Then the biochip was placed in a humidity box overnight at 4^{o}C. Afterwards, the chip was placed inside of a conical tube (containing 7 mL of 1 M ethanolamine) and incubated on a shaking platform for about 1 hour. Subsequently ethanolamine solution was removed without disturbing the active chip area and the chip was washed two times with PBS and 0.5% triton X-100 at room temperature for 5 min while shaking. Finally, the chip was exposed to 1 μL of sample and 0.5 μL $5 \cdot 10^{-6}$ M 4-OHE$_2$-1- N3Ade-SAMSA conjugate, i.e. a fluorescent reporter molecule. The required concentration of 4-OHE$_2$-1-N3Ade-SAMSA conjugate was established in part based on the calibration curve and the results obtained from CE/FASS experiment. After incubation and careful removal of non-bound analytes, 5 μL PBS was added and pipetted up and down at least 15 times. This washing procedure was repeated several times. To keep the chips wet, 2 μL of fresh PBS buffer was added to each spot and the signal was measured by fluorescence imaging using Leica microscope (Germany) equipped with a UV lamp and CCD camera. The concentration of estrogen-derived DNA adducts was estimated form the calibration curve (data not shown).

Measurement of Estradiol Metabolites: LCMS analyses were carried out with a Waters Acquity ultra performance liquid chromatography (UPLC) system connected with a high performance Quattro Micro triple quadrupole mass spectrometer designed for LC/MS-MS operation. Analytical separations on the UPLC system were conducted using an Acquity UPLC BEH C18 1.7 μ column (1 X 100 mm) at a flow rate of 0.15 ml/min. The gradient started with 80% A (0.1% formic acid in H_2O) and 20% B (0.1% formic acid in CH_3CN), changed to 79% A over 4 min, followed by a 6-min linear gradient to 45% A, resulting in a total separation time of 10 min. The elutions from the UPLC column were introduced to the Quatro Micro mass spectrometer.

The ionization method used for the MS analysis was ESI in both the positive (PI) and negative (NI) ion mode with an ESI-MS capillary voltage of 3.0 kV, an extractor cone voltage of 3 V, and a detector voltage of 650 V. Desolvation gas flow was maintained at 600 L/h. Cone gas flow was set at 60 L/h. Desolvation temperature and source temperature were set to 200 and 100 °C, respectively. MS-MS was performed in a multiple reaction monitoring (MRM) mode to produce structural information about the analytes by fragmenting the parent ions inside the mass spectrometer and identifying the resulting daughter/fragment ions. The resulting data were processed by using QuanLynx software (Waters) to quantify the estrogen metabolites. Pure standards were used to optimize the LC/MS conditions prior to analysis.

Statistical methods. The data describing mouse age at the time of tumor appearance (i.e. first clearly palpable tumors) utilized Kaplan-Meier analyses. The various plots were compared to each other using a life table test to determine whether the rate of tumor development was statistically different among the various treatment groups. This analysis takes into account the time a mouse remains on study tumor free and also accounts for animals removed from study due to sickness.

C. Key research accomplishments:

Estrogen receptor independent effects on tumor development

Removal of the ovaries before day 16 in the ERKO animals allowed examination of the role of estradiol acting in the absence of ERα. This ablative procedure appeared to delay the 50% incidence time point to 24 months and reduced the number of animals with tumors by half relative to intact ERKO mice but this trend was not statistically significant (Fig. 1). In addition to E_2, the ovary produces progesterone and inhibins as well as other factors that could confound our results. Accordingly, we examined the dose response effects of E_2 on tumor formation in castrate animals (22,31). Castrate ERKO/Wnt-1 animals received E_2 over a 24 month period and utilized the "estradiol clamp" method to maintain plasma E_2 levels at 5, 10, 80 and 240 pg/ml. The 240 pg/ml dose in the castrate animals caused tumors to develop earlier with a 50% incidence time point at 10 months versus 24 months in vehicle treated animals **(p=0.0002)** (Fig. 2). As in the intact animals, nearly 100% of E_2 treated animals developed tumors. The 80 pg/ml estradiol dose produced effects intermediate between those of vehicle and 240 pg/ml E_2. The 5 and 10 pg/ml E_2 doses appeared to be sub-threshold with no differences compared to vehicle (data not shown).

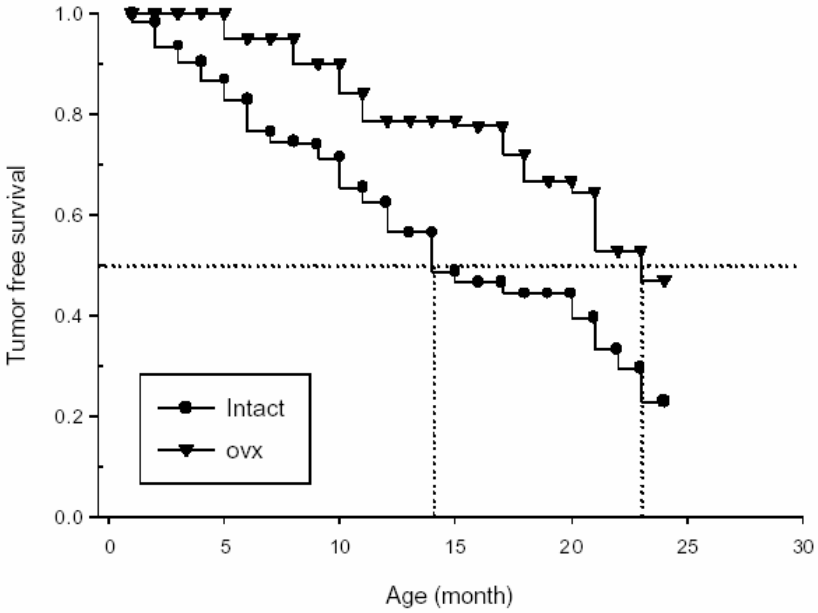

Figure 1. Kaplan –Meyer plots of tumor formation in intact and oophorectecomized (OVX) estrogen receptor knock out (ERKO) transgenic mice.

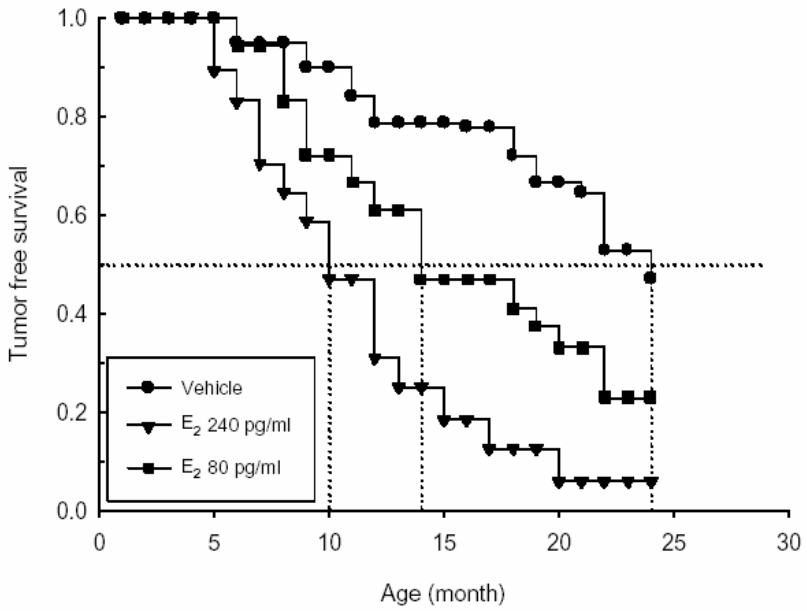

Figure 2. Tumor curves plotted by Kaplan–Meyer analysis in ERKO castrate animals treated with vehicle and with silastic implants delivering estradiol to "clamp" estradiol levels at 80 and 240 pg/ml.

Presence of genotoxic metabolites in mammary tissue: Our working hypothesis suggested that the ER independent effects of E_2 resulted from production of genotoxic metabolites. In order to demonstrate these metabolites in breast tissue, we utilized LC/tandem mass spectrometry methodology to assay the levels of multiple metabolites. In order to enhance the sensitivity of detecting these compounds, we utilized aromatase transfected mice, whose breast tissues contain increased levels of estrogens compared to wild type. The metabolites measured demonstrated the presence of 2- and 4-hydroxylated estrogens, the estradiol quinones, and the depurinated products, 1-N-3-estradiol-adenine and 1-N-7-estradiol-guanine. The 4-OH –estrogen metabolites are shown in Figure 3 and the depurinated compounds in Figure 4. The full range of genotoxic metabolites in ERKO/Wnt-1 tumors are shown in Figure 5. Figure 6 illustrates the levels of the 1-N3-adenine-estrogen depurinated compound as measured by two separate methods to verify its presence in breast tumors.

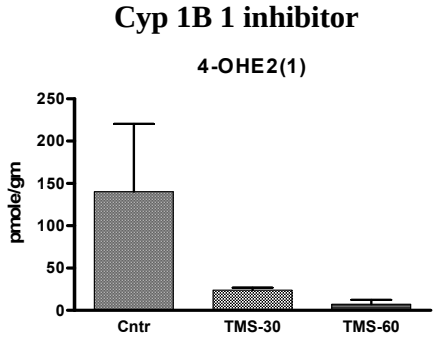

Figure 3. Levels of 4-OH estradiol (E_2) and estrogen (E_1) in breast tissue of aromatase over-expressing transgenic mice. Shown are basal levels and the levels detected during administration of the CYP 1B1 inhibitor, tetra-methoxy-stilbene (TMS) at 30 mg/kg and 60 mg/kg. CYP 1B1 catalyzes the conversion of estradiol and estrone to their 4-OH catecholestrogen metabolites. The inhibition of the 4-OH catecholestrogens provides evidence of the specificity and sensitivity of the assay used.

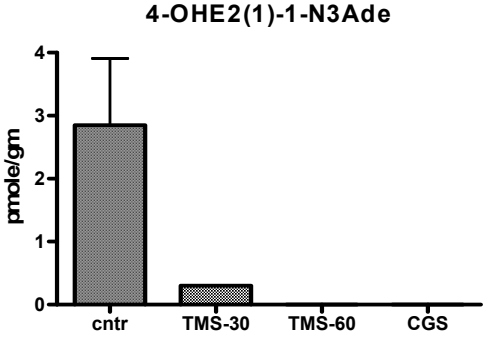

Figure 4. Levels of 4-OH-E_2/E_1-1-N3-adenine in breast tissue of aromatase overexpressing transgenic mice under basal conditions and during administration of TMS at the same doses as above.

C

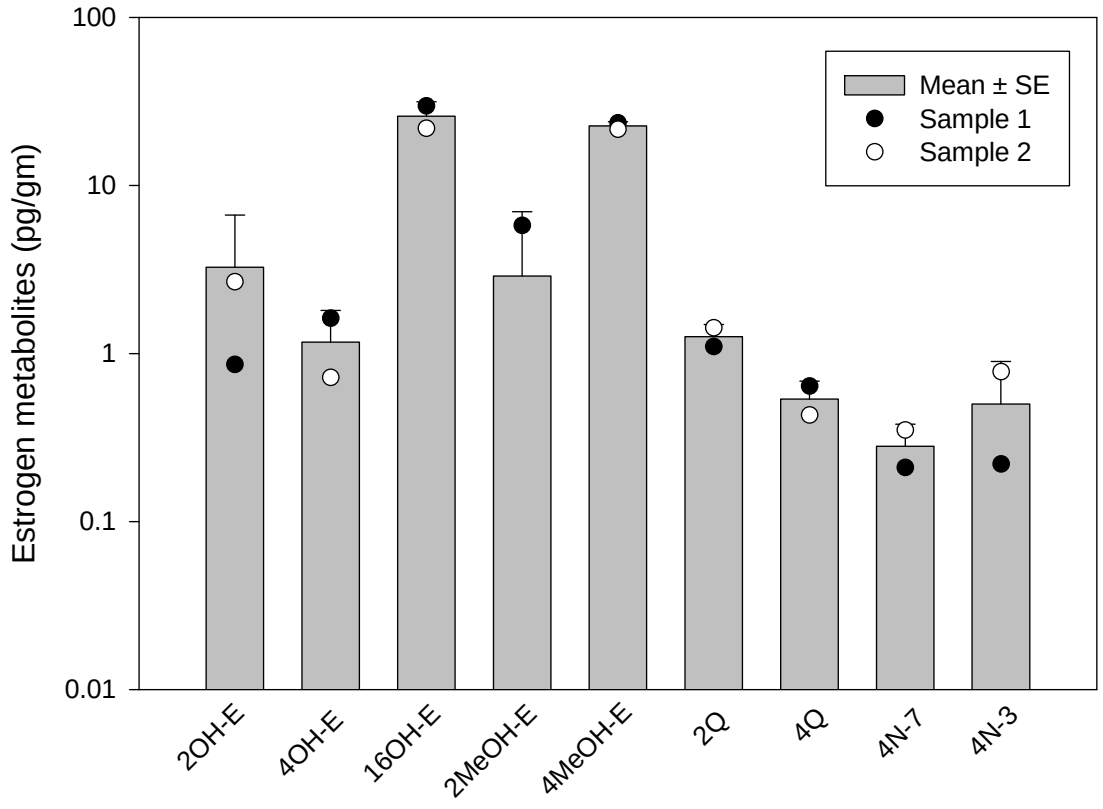

Figure 5. Levels of each of the estrogen metabolites in ERKO/Wnt-1 tumors as measured by LC-tandem mass spectrometry.

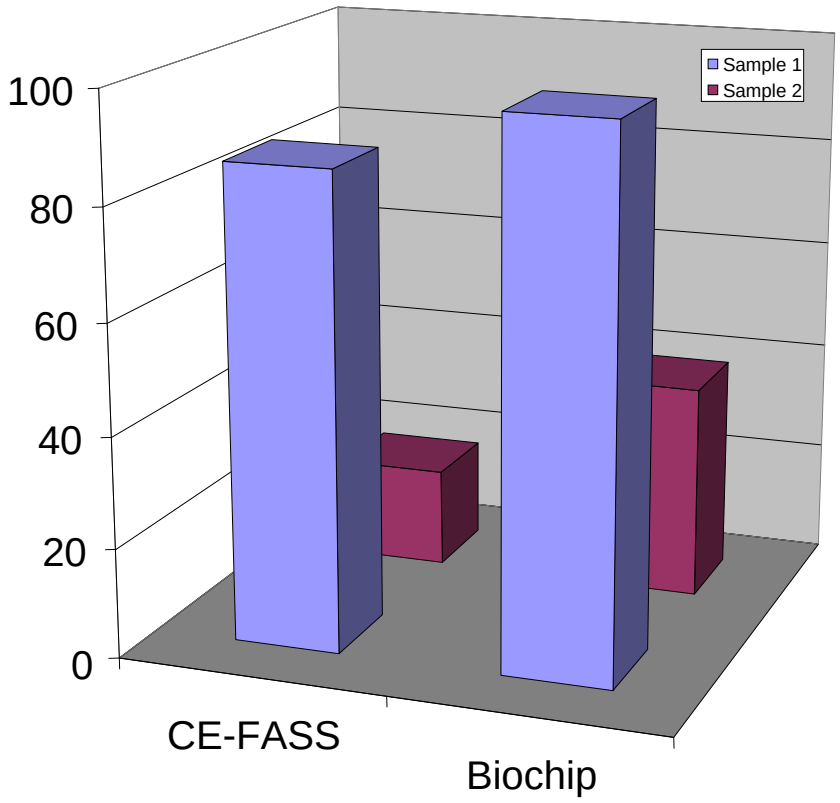

Figure 6. Verification of presence of 1-N3-adenine-estradiol in ERKO/Wnt-1 breast tumors using the CE-FASS and Biochip methods.

Complete elimination of effects of truncated ERα and ERβ. Prior studies had shown that our ERKO mice express an mRNA species that yields a 56 Kd truncated ERα message (32), whose translated protein should retain its DNA and ligand binding domains. However, we had found evidence of only minimal residual ER bio-activity with retention of 1-9% of ER binding activity in uterine cytosols and only 10-20% of stimulation of uterine weight. In our current study, western blot analysis in the ERKO/Wnt-1 animals detected only small amounts of truncated receptor protein. ERβ, while not found on RNase protection assays, could be also be detected by the more sensitive quantitative PCR methodology (33). Recognizing the need to eliminate the biologic effects of these possible residual receptors, we completely abrogated ERα and β function with the "pure antiestrogen" fulvestrant and examined the effect of E_2 under these conditions. Fulvestrant or vehicle was administered to castrate ERKO/Wnt-1 animals with E_2 clamped at 240 pg/ml. The tumors appeared at the same rate in the presence or absence of fulvestrant, providing evidence that the effect of E_2 was not mediated by a truncated ERα or low level ERβ (data not shown). A further strategy abrogated receptor mediated effects by the administration of 17α-OH-E2. This compound lacked ER mediated effects on uterine weight but is capable of forming potentially genotoxic compounds (34). 17α–OH estradiol induced tumors in the ERKO animals at a rate similar to that in animals with E_2 maintained at the same plasma level (i.e. 240 pg/ml) (data not shown) .

Bioassay of "clamped" E_2 on uterine weight. Our various strategies to examine the ER independent effects of E_2 critically depended upon complete blockade of any residual ER activity resulting from a truncated ERα or low level ERβ. Measurement of uterine weight provided a robust bioassay of the tissue effects of E_2 to determine if complete blockade was achieved. We measured uterine weight after at least two months of E_2 exposure under each experimental condition. Uterine weights in the intact (i.e. non-castrate) ER+ /Wnt-1 animals were 84 ± 12 mg (mean ± SE) and in the ER-/Wnt-1, 28 ± 3 mg. Ovariectomy (ovx) reduced uterine weights to 5 ± 0.9 and 4 ± 0.5 mg respectively in both the ER+ and ER- animals. This observation confirmed that the ERKO/Wnt-1 animals did in fact have biologically functional truncated ER alpha. (i.e. difference between intact and castrate ERKO). Administration of E_2 by the "clamp" method increased uterine weight to 164 ± 5 mg in the ER+/Wnt-1 animals and to 24 ± 3 mg in the ER-/Wnt-1. This blunted increase in uterine weight in ERKO animals represented only 18% responsiveness compared to ER+ animals and further confirmed the reduced biologic activity of the truncated E_1 receptor.

Since truncated ER was present and somewhat active, it appeared critically important to demonstrate that fulvestrant completely abrogated its activity. Notably, the administration of fulvestrant completely blocked the residual ER responsiveness in the ERKO/Wnt-1 animals since uterine weight fell to 7 ± 1 mg in the animals receiving this anti-estrogen plus 240 pg/ml E2. Fulvestrant also blocked uterine weight in the E_2 treated ER+/Wnt-1 animals by approximately the same percentage (i.e. 164 to 22 mg or 83% reduction) as in ERKO animals but the absolute reduction in uterine weight was less (22 mg). In aggregate, these data demonstrated that fulvestrant was capable of completely abrogating the effects of residual ER activity in ERKO animals.

We wished to confirm by bioassay that 17α-OH-E_2 also did not stimulate the uterus. At a level of 240 pg/ml this compound caused no increase in uterine weight (4 ± 0.5 mg) indicating its lack of uterotropic activity. As further proof of the minimal ER mediated effects of this compound, we tested its ability to stimulate transcription of endogenous and exogenous estrogen responsive genes and MCF-7 cell growth in vitro. The potency of 17α-OH E_2 on transcription of ERE-luciferase construct (exogenous reporter gene), on progesterone receptor synthesis (endogenous genes), and on cell growth was 1% or less than that of E_2 itself (data not shown).

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Manuscripts Accepted

1. The Breast Cancer Prevention Collaborative Group: Richard J. Santen, Norman F. Boyd, Rowan T. Chlebowski, Steven Cummings, John F. Cuzick, Mitch Dowsett, Douglas Easton, John F. Forbes, Tim Key, Susan E. Hankinson, Anthony Howell, and James Ingle. Critical assessment of risk factors for breast cancer: what should be used to develop a new breast cancer prediction model? In Press Endocrine related Cancer 2007

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E. Conclusions

In summary, these studies provide proof of the principle that breast cancer development can be influenced by ER independent as well as ER dependent mechanisms and that these effects act in concert. Accordingly, this study provides mechanistic evidence in support of the use of aromatase inhibitors in preference to the anti-estrogens

for prevention of breast cancer. Anti-estrogens primarily block receptor mediated pathways whereas the aromatase inhibitors block both receptor mediated and receptor independent effects of estradiol. Two current and one planned clinical trials are examining the aromatase inhibitors for prevention of breast cancer (35,36).

SPECIFIC AIM 5 – INGLE

A. Introduction

Globally, breast cancer remains a major problem for many women and is a concern for virtually all women. Much of the clinical research to date has involved large and very expensive clinical trials. The most recently reported is that of the Study of Tamoxifen and Raloxifene (STAR) that involved over 19,000 women accrued over six years with a reported cost of about $118,000,000. The current major efforts in prevention involve placebo-controlled trials of exemestane (MAP.3) involving almost 5,000 patients, and anastrazole (IBIS 2) that involves 6,000 women. It is clear that new approaches and paradigms are needed to identify biologically-based strategies for prevention of breast cancer. It would be of great value to identify biomarkers that would allow targeted studies involving smaller sample sizes at much lower cost.

B. Body

The approach taken in Specific Aim 5 has been to develop and conduct a prospective study aimed at determining levels of estrogen, catechol estrogen metabolites, catechol estrogen-glutathione conjugates, and most importantly, catechol estrogen-DNA adducts in women at high risk of developing breast cancer and in women with a personal history of breast cancer. The protocol that was developed at Mayo Clinic is entitled “Estrogen-DNA Adducts in Breast, Urine, and Serum as Biomarkers of Breast Cancer Risk” (protocol #19-2005).

To date material has been collected on 58 high risk women and 24 women with newly diagnosed breast cancer. An amendment has recently been approved by the Mayo Institutional Review Board to collect specimens (nipple aspirate fluid, urine, and serum) on low or average risk women. The amended protocol is appended and will be formally activated by the end of May 2007. The goal is to collect specimens on a total of 300 women (100 each from high-risk women, women with recently diagnosed breast cancer, and low or average risk women).

C. Key Research Accomplishments

Initial results have been developed from 12 high risk women and 17 women with a personal history of breast cancer from Mayo that have been combined with 4 average risk women from the University of Nebraska combined with 42 such women from Italy. Analysis of estrogen metabolites, conjugates, and depurinating DNA adducts in urine samples from these 46 healthy control women, 12 high-risk women, and 17 women with breast cancer showed that the levels of the ratios of depurinating DNA adducts to their respective estrogen metabolites and conjugates were significantly higher in high-risk women ($p<0.001$) and women with breast cancer ($p<0.001$) than control subjects. The high-risk and breast cancer groups were not significantly different ($p=0.62$). After adjusting for patient characteristics, these ratios were still significantly associated with

health status [1]. These findings support the hypothesis that depurinating estrogen-DNA adducts can serve as potential biomarkers of risk of developing breast cancer.

A second study in which urine and serum samples were collected from 40 control women, 40 women at high risk for breast cancer and 40 women with breast cancer, has been completed. Two manuscripts have been submitted for publication [2,3].

D. Reportable Outcomes

1. Gaikwad, N.W., Yang, L., Muti, P., Meza, J.L., Pruthi, S., Ingle, J.N., Rogan, E.G. and Cavalieri, E.L. The molecular etiology of breast cancer: evidence from biomarkers of risk. Int. J. Cancer, 122:1949-1957, 2008.

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E. Conclusions

The results to date, as noted above, are highly supportive of the hypothesis that estrogen genotoxicity plays an important role in breast cancer development. Of particular importance is that depurinating estrogen-DNA adducts appear to biomarkers of risk and we are continuing the study of this concept with expansion of our clinical samples.

F. References

None to date

Int. J. Cancer: **122,** 1949–1957 (2008)

The molecular etiology of breast cancer: Evidence from biomarkers of risk

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Estrogens can become endogenous carcinogens *via* formation of catechol estrogen quinones, which react with DNA to form specific depurinating estrogen-DNA adducts. The mutations resulting from these adducts can lead to cell transformation and the initiation of breast cancer. Estrogen metabolites, conjugates and depurinating DNA adducts in urine samples from 46 healthy control women, 12 high-risk women and 17 women with breast cancer were analyzed. The estrogen metabolites, conjugates and depurinating DNA adducts were identified and quantified by using ultra-performance liquid chromatography/tandem mass spectrometry. The levels of the ratios of depurinating DNA adducts to their respective estrogen metabolites and conjugates were significantly higher in high-risk women ($p < 0.001$) and women with breast cancer ($p < 0.001$) than in control subjects. The high-risk and breast cancer groups were not significantly different ($p = 0.62$). After adjusting for patient characteristics, these ratios were still significantly associated with health status. Thus, the depurinating estrogen-DNA adducts are possible biomarkers for early detection of breast cancer risk and response to preventive treatment.

Key words: breast cancer risk; depurinating estrogen-DNA adducts; estrogen biomarkers; balance in estrogen metabolism

Development of noninvasive tests of breast cancer risk has been a major goal for more than 30 years. In this article we present biomarkers of risk that are related to the hypothesized first critical step in the initiation of breast cancer, namely, the reaction of catechol estrogen quinone metabolites with DNA.[1] Prevention of cancer can be achieved by blocking this DNA damage, which generates the mutations leading to the initiation, promotion and progression of cancer.[2]

Exposure to estrogens is a known risk factor for breast cancer.[3,4] The discovery that specific oxidative metabolites of estrogens, namely, catechol estrogen quinones, can react with DNA[5–9] led to and supports the hypothesis that these metabolites can become endogenous chemical carcinogens. Some of the mutations generated by this specific DNA damage can result in the initiation of cancer.[1,5] This paradigm suggests that specific, critical mutations generate abnormal cell proliferation leading to cancer.[1,10–13]

As illustrated in Figure 1, in the metabolism of catechol estrogens there are activating pathways[14] that lead to the formation of the estrogen quinones, estrone (estradiol) quinones [$E_1(E_2)$-Q], which can react with DNA. There are also deactivating pathways that limit formation of the quinones and/or prevent their reaction with DNA. These are methylation of catechol estrogens,[15] conjugation of the $E_1(E_2)$-Q with glutathione (GSH)[16] and reduction of the quinones to catechols[17] (Fig. 1).

When $E_1(E_2)$-3,4-Q react with DNA, they form predominantly the depurinating adducts 4-hydroxyestrone(estradiol)-1-N3Adenine [4-$OHE_1(E_2)$-1-N3Ade] and 4-hydroxyestrone(estradiol)-1-N7Guanine [4-$OHE_1(E_2)$-1-N7Gua],[5–7] whereas $E_1(E_2)$-2,3-Q form much lower levels of 2-hydroxyestrone(estradiol)-6-N3Adenine [2-$OHE_1(E_2)$-6-N3Ade] (Figs. 1 and 2).[7] Both $E_1(E_2)$-3,4-Q and $E_1(E_2)$-2,3-Q form much lower levels of stable DNA adducts than depurinating adducts.[5–7] Once released from the DNA, the depurinating estrogen-DNA adducts are shed from cells into the bloodstream and, eventually, are excreted in urine.

The release of the depurinating adducts generates apurinic sites in DNA, which in turn, may induce mutations. The observation of Harvey-*ras* mutations within 6–12 hr after treatment of mouse skin or rat mammary glands with E_2-3,4-Q suggests that these mutations arise *via* error-prone base excision repair.[1,10,11] Similar patterns of mutations have also been observed in the big blue (BB) rat mammary gland and cultured BB rat2 embryonic cells after treatment with 4-hydroxyestradiol (4-OHE_2) or E_2-3,4-Q.[1,12] The transforming activity of E_2 and 4-OHE_2 has been observed in human breast epithelial (MCF-10F) cells, which do not contain estrogen receptor-α, and it is not affected by the presence of an anti-estrogen.[18–20] Furthermore, 4-$OHE_1(E_2)$ are carcinogenic in the Syrian golden hamster and CD-1 mouse.[21–24] All of these results support the hypothesis that estrogens initiate cancer through their genotoxicity.

Initiation of cancer by estrogens is based on estrogen metabolism in which the homeostatic balance between activating and deactivating pathways is disrupted (Fig. 1). Activating pathways are the ones that oxidize E_1 and E_2 to their catechol estrogen quinones, whereas the deactivating pathways are the ones that block oxidation.[1] A variety of factors, such as diet, environment and lifestyle, can unbalance the equilibrium between these 2 pathways. When estrogen metabolism is balanced, the level of estrogen-DNA adducts in tissue and urine is low and/or the levels of estrogen metabolites and conjugates are high. In contrast, when estrogen metabolism is unbalanced, the level of DNA adducts in tissue and urine is high and/or the levels of estrogen metabolites and conjugates are low. It is this imbalance in estrogen metabolism, leading to relatively high levels of estrogen-DNA adducts, that may be a critical determinant of breast cancer initiation.

The above considerations led us to hypothesize that estrogen metabolites, conjugates and depurinating DNA adducts may differ between healthy women and women with breast cancer or at high risk of breast cancer. To test this hypothesis, we conducted a cross-sectional study in which 40 estrogen metabolites, conjugates and depurinating DNA adducts were analyzed in urine samples from healthy women, women at high risk for breast cancer based

Abbreviations: Cys, cysteine; ESI, electrospray ionization; $E_1(E_2)$-Q, estrone(estradiol)-quinones; GSH, glutathione; 4-OHE_2, 4-hydroxyestradiol; 4-$OHE_1(E_2)$-1-N3Ade, 4-hydroxyestrone(estradiol)-1-N3Adenine; 4-$OHE_1(E_2)$-1-N7Gua, 4-hydroxyestrone(estradiol)-1-N7Guanine; 2-$OHE_1(E_2)$-6-N3Ade, 2-hydroxyestrone(estradiol)-6-N3Adenine; MRM, multiple reaction monitoring; NAcCys, *N*-acetylcysteine; NI, negative ion; PI, positive ion; SPE, solid-phase extraction; UPLC/MS-MS, ultraperformance liquid chromatography/tandem mass spectrometry.

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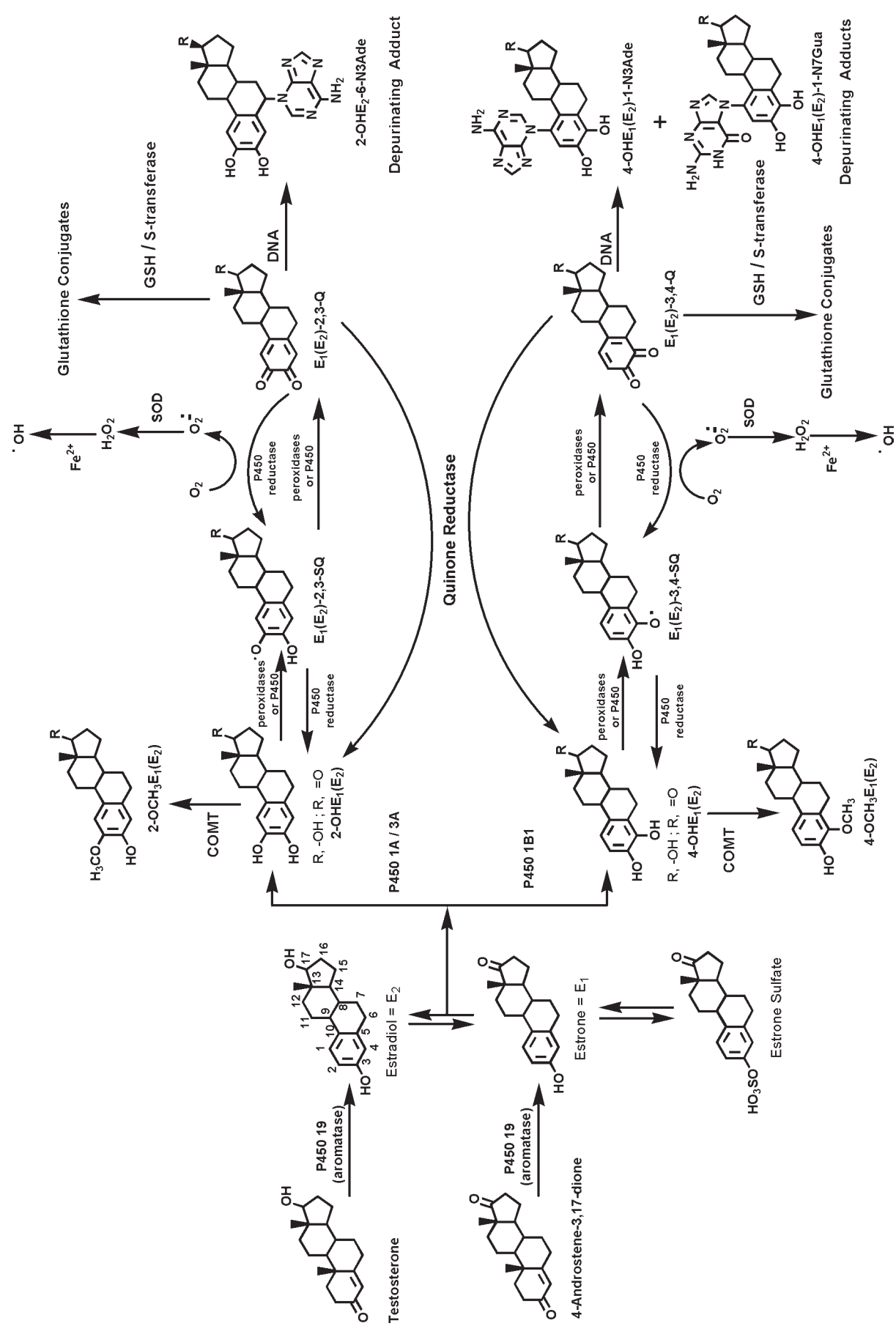

FIGURE 1 – Biosynthesis and metabolic activation of the estrogens, E_1 and E_2. The metabolic activation of E_1 and E_2 leads to 2- and 4-catechol derivatives, which further oxidize to yield the corresponding reactive quinones. The quinones react with DNA to form depurinating DNA adducts. In the deactivation pathway, which operates in parallel, the catechol derivatives are methylated to form methoxy catechol estrogens; in addition, the quinones are reduced by quinone reductase, as well as are conjugated with GSH, and, thus, are rendered harmless. The shift in the apparent balance between these activating and deactivating pathways towards formation of depurinating DNA adducts could lead to the initiation of breast cancer.

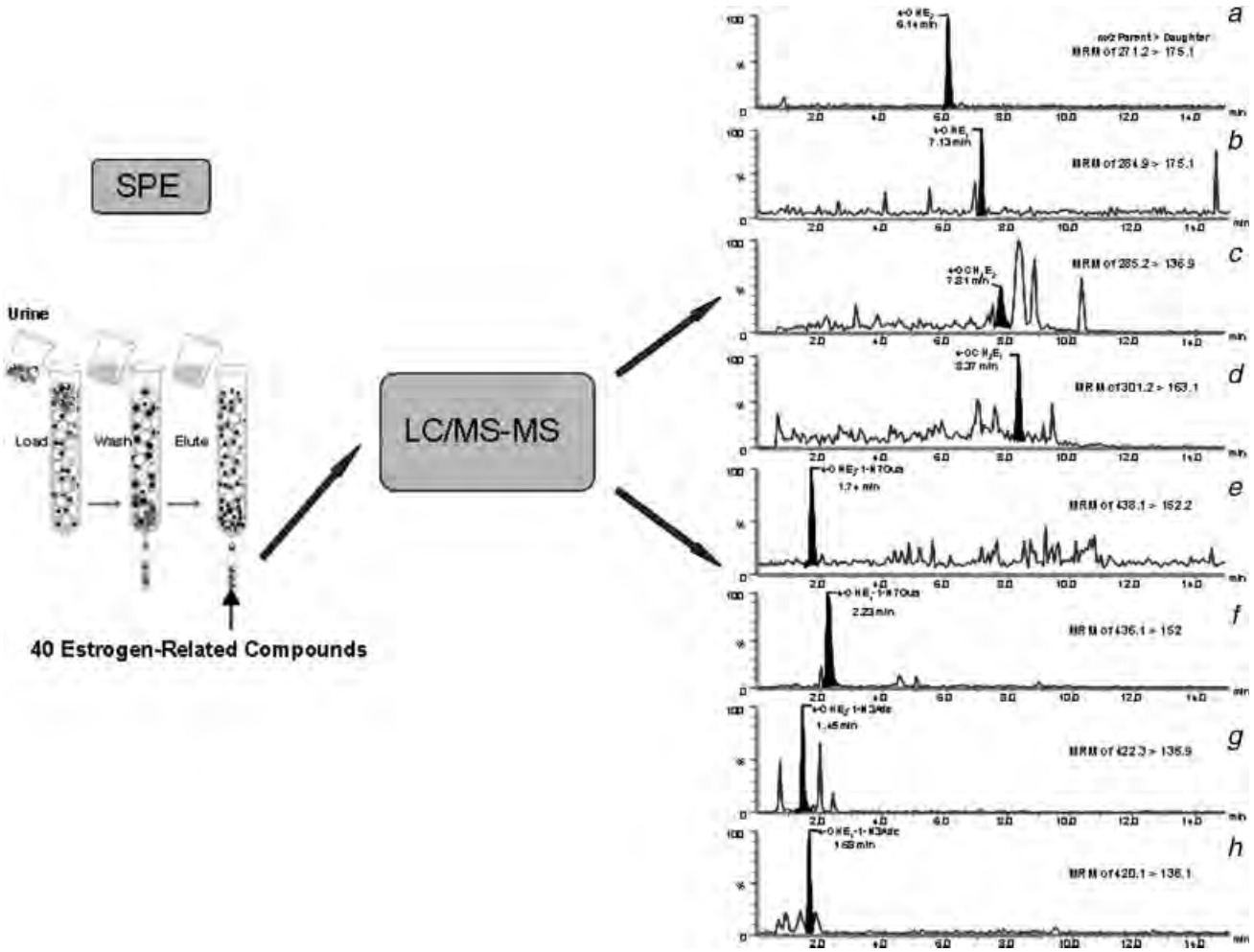

FIGURE 2 – Schematic representation of the steps carried out to purify by SPE and analyze by UPLC/MS-MS the estrogen-related compounds from urine samples. The UPLC/MS-MS chromatograms of (*a*) 4-OHE_2, (*b*) 4-OHE_1, (*c*) 4-OCH_3E_2, (*d*) 4-OCH_3E_1, (*e*) 4-OHE_2-1-N7Gua, (*f*) 4-OHE_1-1-N7Gua, (*g*) 4-OHE_2-1-N3Ade and (*h*) 4-OHE_1-1-N3Ade that are shown in the figure are representatives from the 40 different estrogen-related compounds seen in the urine samples.

on Gail Model score >1.66%, and women with breast carcinoma. The Gail Model takes into account the following factors: age, age at menarche, age at first live birth, number of breast biopsies and history of atypical hyperplasia, number of first degree relatives with breast cancer (mother, sister and daughter) and race. A 5-year Gail Model score of >1.66% is considered high risk.[25]

Material and methods

Materials

Phenyl solid phase extraction (SPE) cartridges were purchased from Varian (Palo Alto, CA). Androstenedione (**1**), (Table I), testosterone (**2**), estrone (E_1) sulfate (**3**), E_2 (**4**), E_1 (**5**), 2-OHE_2 (**6**), 2-OHE_1 (**7**), 16α-OHE_2 (**10**), 16α-OHE_1 (**11**), 2-OCH_3E_2 (**12**), 2-OCH_3E_1 (**13**), 4-OCH_3E_2 (**14**), 4-OCH_3E_1 (**15**), 2-OH-3-OCH_3E_2 (**16**) and 2-OH-3-OCH_3E_1 (**17**) were purchased from Steraloids (Newport, RI). 4-OHE_2 (**8**) and 4-OHE_1 (**9**) were synthesized as previously described.[26] 2-OHE_2-1-SG (**18**), 2-OHE_2-4-SG (**19**), 2-OHE_1-1-SG (**20**), 2-OHE_1-4-SG (**21**), 2-OHE_2-(1+4)-Cys (**22**), 2-OHE_1-1-Cys (**23**), 2-OHE_1-4-Cys (**24**), 2-OHE_2-1-NAcCys (**25**), 2-OHE_2-4-NAcCys (**26**), 2-OHE_1-1-NAcCys (**27**), 2-OHE_1-4-NAcCys (**28**), 4-OHE_2-2-SG (**29**), 4-OHE_1-2-SG (**30**), 4-OHE_2-2-Cys (**31**), 4-OHE_1-2-Cys (**32**), 4-OHE_2-2-NAcCys (**33**) and 4-OHE_1-2-NAcCys (**34**) were synthesized by using the procedure of Cao *et al.*[27] 4-OHE_2-1-N7Gua (**35**), 4-OHE_1-1-N7Gua (**36**), 4-OHE_2-1-N3Ade (**37**), 4-OHE_1-1-N3Ade (**38**), 2-OHE_2-6-N3Ade (**39**) and 2-OHE_1-6-N3Ade (**40**) were synthesized by following the reported methods.[6,7,28] All solvents were HPLC grade and all other chemicals used were of the highest grade available.

Study population

We collected urine from 75 women at 3 different sites: (*i*) at the Center for Mammographic Screening at the University of Naples, Italy (42 women), (*ii*) at the Breast Diagnostic Clinic and Oncology Breast Clinic of the Mayo Clinic, Rochester, MN (18 women) and (*iii*) at the Olson Center for Women's Health, University of Nebraska Medical Center (UNMC), Omaha, NE (15 women). Women were recruited between March 2005 and September 2006 and their ages ranged between 34 and 73 years—healthy women: range, 34–67; mean, 50 ± 8; high-risk women: range, 44–64; mean, 52 ± 6; women with breast cancer: range, 34–73; mean, 54 ± 10.

All women recruited at the University of Naples were healthy (they did not receive a diagnosis of breast cancer at the time of their mammographic test). Among the women recruited at the Mayo Clinic, 12 were classified as high-risk women (Gail Model score = 1.67%–11.7%) and 6 were breast cancer cases. At UNMC, 4 women were healthy, that is, had no known cancer, and 11 were diagnosed with breast cancer. None of the subjects received estrogen-containing treatment for at least 3 months prior

TABLE I – MASS SPECTROMETRIC PARAMETERS[1]

No.	Compound	Mass	ESI mode	Parent (*m/z*)	Daughters (*m/z*)	Cone (volt)	Collision	Retention time	LOD (fmol)
1	Androstenedione	286.2	Positive	287.1	97.1	40	19	8.43	14
2	Testosterone	288.2	Positive	289.2	97.0	40	19	7.97	35
3	E_1-Sulfate	350.1	Negative	249.0	269.0	50	28	6.61	143
4	E_2	272.4	Positive	273.2	135.2	30	14	7.74	184
5	E_1	270.1	Positive	271.2	253.2	25	14	8.43	148
6	2-OHE_2	288.2	Positive	271.2	175.1	30	14	6.74	69
7	2-OHE_1	286.2	Negative	285.0	160.9	65	37	7.3	18
8	4-OHE_2	288.2	Positive	271.2	175.1	30	14	6.14	69
9	4-OHE_1	286.2	Negative	284.9	161.0, 175.1	65	35	7.13	35
10	16α-OHE_2	288.4	Positive	289.0	107.0	25	14	2.42	867
11	16α-OHE_1	286.4	Negative	285.1	145.1	30	15	4.67	349
12	2-OCH_3E_2	302.2	Positive	285.2	136.9, 189.1	32	15	8.25	330
13	2-OCH_3E_1	300.2	Positive	301.2	136.9, 163.1	30	17	8.85	333
14	4-OCH_3E_2	302.2	Positive	285.2	136.9, 189.1	32	15	7.81	66
15	4-OCH_3E_1	300.2	Positive	301.2	163.1, 283.1	30	17	8.37	133
16	2-OH-3-OCH_3E_2	302.4	Positive	285.2	189.1	32	15	8.71	165
17	2-OH-3-OCH_3E_1	300.4	Positive	301.2	163.1	30	17	9.07	33
18	2-OHE_2-1-SG	593.7	Positive	594.1	319.1, 465.0	42	20	1.72	8.4
19	2-OHE_2-4-SG	593.7	Positive	594.0	319.1, 465.4	35	21	2.32	8.4
20	2-OHE_1-1-SG	591.0	Positive	592.1	316.8	45	22	2.65	1.7
21	2-OHE_1-4-SG	591.0	Positive	592.2	317.1, 463.2	40	22	2.65	1.7
22	2-OHE_2-1+4-Cys	407.2	Positive	408.2	319.0	30	17	1.73	12
23	2-OHE_1-1-Cys	405.2	Positive	406.0	316.9	35	15	3.25	6.2
24	2-OHE_1-4-Cys	405.2	Positive	406.2	317.1	30	17	3.25	6.2
25	2-OHE_2-1-NAcCys	449.2	Positive	450.1	162.0, 287.4	25	14	4.07	5.6
26	2-OHE_2-4-NAcCys	449.2	Positive	450.2	162.0, 287.2	30	14	4.07	5.6
27	2-OHE_1-1-NAcCys	447.2	Positive	448.1	162.0, 285.4	30	13	6.05	5.6
28	2-OHE_1-4-NAcCys	447.2	Positive	448.0	162.0, 284.9	35	14	6.05	5.6
29	4-OHE_2-2-SG	593.2	Positive	594.4	318.9, 464.8	42	20	2.33	8.4
30	4-OHE_1-2-SG	591.2	Positive	592.3	317.1, 462.9	45	22	2.65	8.5
31	4-OHE_2-2-Cys	407.2	Positive	408.0	318.9, 286.1	40	16	2.24	2.4
32	4-OHE_1-2-Cys	405.2	Positive	406.0	316.9, 389.0	35	15	2.84	6.2
33	4-OHE_2-2-NAcCys	449.2	Positive	450.1	162.1	35	15	5.91	5.6
34	4-OHE_1-2-NAcCys	447.2	Positive	448.3	161.8	35	14	6.64	2.2
35	4-OHE_2-1-N7Gua	437.2	Positive	438.1	152.2, 272.0	62	38	1.74	2.3
36	4-OHE_1-1-N7Gua	435.2	Positive	436.1	152.0, 271.9	62	38	2.23	2.2
37	4-OHE_2-1-N3Ade	421.2	Positive	422.3	135.9, 257.1	62	45	1.45	5.9
38	4-OHE_1-1-N3Ade	419.2	Positive	420.1	296.0, 136.1	60	44	1.68	2.4
39	2-OHE_2-6-N3Ade	421.1	Positive	422.2	136.0, 287.0	26	10	1.05	1.2
40	2-OHE_1-6-N3Ade	419.1	Positive	420.0	135.9	26	10	1.41	2.4

[1]List of the 40 estrogen-related compounds with the masses of parent and daughter ions and the ionization mode that were used for MRM method optimization. The last column indicates the limit of detection for each compound.

to providing a urine sample. The 3 groups were frequency matched on age, race and menopausal status.

All procedures were approved by the University of Naples, Mayo Clinic and UNMC Institutional Review Boards. Signed consents included authorization to collect and bank urine samples and collect demographic and clinical information.

Sample collection

A standardized method was followed to collect all of the urine samples. A spot urine sample of about 50 ml was collected from each participant and 1 mg/ml ascorbic acid was added to prevent oxidation of the catechol moieties in the various estrogen compounds. The urine samples were aliquoted, frozen and four 10-ml aliquots were transferred to the Eppley Institute, UNMC, on dry ice and were stored at −80°C until analysis. Thus, each analytical sample was thawed only once prior to analysis.

Solid-phase extraction of urine

Two milliliter aliquots of urine were partially purified by SPE. The SPE was performed using a 20-port SPE vacuum manifold with phenyl cartridges (Fig. 2). Urine samples were adjusted to pH 7 with 1 M NaOH or 1 M HCl. For method development and validation, 2-ml aliquots of charcoal-treated human urine samples were spiked with a total of 250, 500 or 1,000 pg of the 40 estrogen-related compounds (final concentration 0.125, 0.25 and 0.50 pg/μl) and loaded onto the phenyl 100-mg cartridges preconditioned with CH_3OH and the loading buffer, 10 mM ammonium formate, pH 7. The cartridges were washed with the loading buffer, and then the compounds of interest were eluted from the cartridge by using an elution buffer, methanol/10 mM ammonium formate, pH 7 (90:10) with 1% acetic acid. This procedure led to enrichment of the 40 estrogen-related compounds after elution. Charcoal-treated urine (2 ml) was used in controls, and the eluates from the SPE cartridges were spiked with 250, 500 or 1,000 pg of the 40 estrogen-related compounds. The eluates from both the experimental and control samples were concentrated using a SpeedVac and lypholizer, and subjected to ultraperformance liquid chromatography/tandem mass spectrometry (UPLC/MS-MS) analysis. To determine the recovery of the standards by the SPE method, comparison was made between the corresponding concentrations of experimental and control samples (Fig. 3). Study samples were cleaned in duplicate by using the above optimized SPE conditions and analyzed by UPLC/MS-MS.

UPLC/MS-MS analysis of urine samples

The 40 analytes (Table I) included the androgens androstenedione and testosterone; the estrogens E_1 sulfate, E_1 and E_2; the catechol estrogens 2-OHE_1(E_2) and 4-OHE_1(E_2); the 16α-OHE_1(E_2); the methylated 2- and 4-catechol estrogens; the 2- and 4-catechol estrogens conjugated with GSH, cysteine (Cys) or *N*-acetylcys-

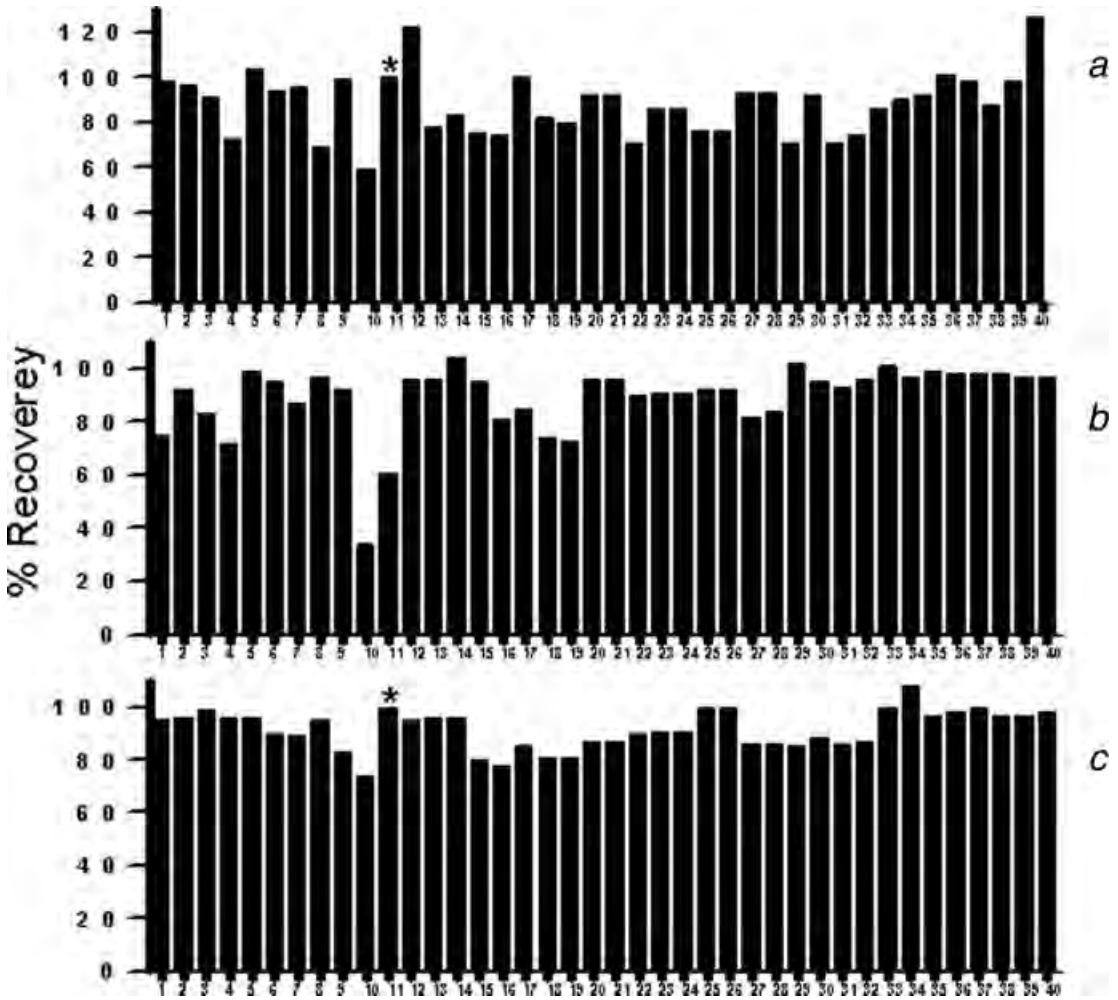

FIGURE 3 – SPE recovery of standard 40 estrogen-related compounds. The 2-ml aliquots of activated charcoal-treated human urine samples were spiked with the total (*a*) 250, (*b*) 500 and (*c*) 1,000 pg of 40 estrogen-related compounds before and after (control) passing over phenyl SPE cartridges. The recovery of each compound was determined by comparing the experimental values to the controls.

teine (NAcCys); and the depurinating DNA adducts of 4-$OHE_1(E_2)$ and 2-$OHE_1(E_2)$. All of the estrogen compounds were analyzed as both E_1 and E_2 derivatives because the interconversion of these 2 estrogens is carried out continuously by 17β-estradiol dehydrogenase.

All experiments were performed on a Waters (Milford, MA) Quattro Micro triple quadrupole mass spectrometer by using electrospray ionization (ESI) in positive ion (PI) and negative ion (NI) mode, with an ESI-MS capillary voltage of 3.0 kV, an extractor cone voltage of 2 V, and a detector voltage of 650 V. Desolvation gas flow was maintained at 600 l/h. Cone gas flow was set at 60 l/h. Desolvation temperature and source temperature were set to 200 and 100°C, respectively. For all the studies, a methanol:-water (1:1) mixture with 0.1% formic acid was used as the carrier solution. ESI interface tuning and mass calibration were accomplished in the PI mode by using a standard sodium iodide-rubidium iodide solution. The test sample (compounds 1 through 40) was introduced to the source at a flow rate of 10 μl/min by using an inbuilt pump. PI or NI detection was used in cases where the sample was readily ionized to cation or anion, respectively. The masses of parent ion and daughter ions were obtained in the MS and MS-MS operations. The parent and daughter ion data obtained for each compound were used to generate the multiple reaction monitoring (MRM) method for UPLC/MS-MS operation (Table I).

Measurements of estrogen-related compounds in urine extracts were conducted by using UPLC/MS-MS. UPLC/MS-MS analyses were carried out with a Waters Acquity UPLC system connected with the high-performance Quattro Micro triple quadrupole mass spectrometer. Analytical separations on the UPLC system were conducted using an Acquity UPLC BEH C18 1.7 μm column (1 × 100 mm) at a flow rate of 0.15 ml/min. The gradient started with 80% A (0.1% formic acid in H_2O) and 20% B (0.1% formic acid in CH_3CN), changed to 79% A over 4 min, followed by a 6-min linear gradient to 45% A, resulting in a total separation time of 10 min. The elutions from the UPLC column were introduced to the Quattro Micro mass spectrometer.

The ionization method used for MS analysis was ESI in both the PI and NI mode. MS-MS was performed in the MRM mode (see above), and resulting data were processed by using QuanLynx software (Waters) to quantify the estrogen metabolites. To calculate limits of detection, various concentrations, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10, 25, 50 and 100 pg/μl, of the analyte were injected to UPLC/MS-MS. The injected amount that resulted in a peak with a height at least 2 or 3 times as high as the baseline noise level was used as the limit of detection (Table I). Pure standards were used to optimize the UPLC/MS conditions prior to analysis. After UPLC analysis, the mean value was calculated for all the compounds obtained from each sample.

Statistical methods

Estrogen-related compounds were compared for control *versus* high risk and for control versus breast cancer using a Mann–Whitney test, with *p*-values adjusted for the 2 multiple comparisons using the Bonferroni method. To account for the multiple hypothesis tests conducted for these variables, a *p*-value <0.01 was interpreted as statistically significant. The log-transformed sum of the ratios of depurinating adducts to the corresponding metabolites and conjugates was compared using a one way ANOVA, and *post hoc* comparisons were made using the method of Bonferroni. Linear regression was used to assess the association between disease status and ratio adjusted for age at recruitment, age at menarche, menopausal status (categorical) and parity for the 56 subjects with patient characteristics available. All the statistics and *p*-values were calculated using SPSS software (SPSS, Chicago, IL).

Results and discussion

Analysis of urine samples

After partial purification of the urine samples by SPE (Fig. 2), we analyzed the 40 estrogen-related compounds using UPLC/MS-MS. The advantage of having MS detector in MRM mode over conventional high pressure liquid chromatography analysis is that number of channels in the detector could be set to specifically and separately identify all the estrogen related compounds (Fig. 2). Each metabolite was detected and identified based on the parameters that are unique to them, such as mass (parent and daughter), retention time and ionization mode (positive and negative) (Table I). The typical spectra of representative estrogen derivatives, which were obtained in a single injection, are shown in Figure 2. The levels of estrogen-related compounds for a high risk woman, measured from single injection, are presented in Table II.

Treatment of urine with glucuronidase/sulfatase led to significant increases (10 to 20-fold) in the levels of E_1 and E_2, while the levels of estrogen metabolites, conjugates and adducts changed marginally and in many cases decreased because of the incubation for 8 hr at 37°C. To avoid artifacts and errors that are introduced by maintaining the urine samples at 37°C for 8 hr, we carried out all the analyses without glucuronidase/sulfatase treatment. Therefore, the observed levels of E_1 and E_2, as reported in Table II, for example, were 10 to 20-fold lower than the total values. Since estrone and estradiol are constantly inter-converting, we have combined estrone and estradiol values of all the derivatives (Tables II and III). The GSH conjugates of estrogen quinones are further converted to Cys and NAcCys conjugates *via* the mercapturic acid biosynthesis pathway.[29] Hence we have combined all the values of 2 conjugates and 4 conjugates (Tables II and III), which reflect the total protection by GSH from 2 or 4 quinones, respectively. The results presented here clearly demonstrate the ability of SPE combined with UPLC/MS-MS analysis to resolve, identify and quantify 40 estrogen-related compounds with accuracy and speed.

The values obtained for the various estrogen-related compounds in 3 groups of women were processed in 2 different ways. First, median values were calculated for all the compounds and their levels were examined in the 3 groups of women (Table III). Then, we used the ratio of depurinating N3Ade and N7Gua adducts to the sum of their respective estrogen metabolites and conjugates in urine samples because the ratio reflects the degree of imbalance in estrogen metabolism that can lead to cancer initiation (Fig. 4). A high ratio of adducts to their respective metabolites and conjugates represents relatively more DNA damage. In contrast, a low ratio of adducts to their respective metabolites and conjugates means that relatively little of the estrogen metabolites reacted with DNA.

Median values of the urinary estrogen-related compounds in the 3 groups of women

Using the newly developed SPE/UPLC/MS-MS methodology, we have analyzed urine samples of various women's groups for estrogen-related compounds. The data obtained were used to calculate median values for each of the 40 compounds (Table III).

The median andostenedione, testosterone, E_2/E_1, 16α-OHE_2/16α-OHE_1, 4-OCH_3E_2/4-OCH_3E_1, 2-$OHE_1(E_2)$ GSH conjugate and derivative values were higher for controls compared to high

TABLE II – REPRESENTATIVE METABOLIC PROFILE OF A URINE SAMPLE OBTAINED FROM A HIGH RISK WOMAN.[1]

No.	Compound	pmole/mg creatinine mean, $n = 2$	Total pmole/mg creatinine
1	Androstenedione	1.56	1.56
2	Testosterone	0.24	0.24
3	E_1 Sulfate	1.81	1.81
4	E_2[4]	5.29	15.93
5	E_1[4]	10.64	
6	2-OHE_2	3.09	3.15
7	2-OHE_1	0.05	
8	4-OHE_2	2.64	2.91
9	4-OHE_1	0.27	
10	16α-OHE_2	12.12	38.64
11	16α-OHE_1	26.52	
12	2-OCH_3E_2	1.95	49.81
13	2-OCH_3E_1	47.87	
14	4-OCH_3E_2	0.41	5.08
15	4-OCH_3E_1	4.67	
16	2-OH-3-OCH_3E_2	1.91	10.27
17	2-OH-3-OCH_3E_1	8.36	
18	2-OHE_2-1-SG	0.17	3.10[5]
19	2-OHE_2-4-SG	0.17	
20	2-OHE_1-1-SG	0.49	
21	2-OHE_1-4-SG	0.47	
22	2-OHE_2-1+4-Cys	0.27	
23	2-OHE_1-1-Cys	0.10	
24	2-OHE_1-4-Cys	0.44	
25	2-OHE_2-1-NAcCys	0.07	
26	2-OHE_2-4-NAcCys	0.07	
27	2-OHE_1-1-NAcCys	0.43	
28	2-OHE_1-4-NAcCys	0.43	
29	4-OHE_2-2-SG	0.51	1.77[6]
30	4-OHE_1-2-SG	0.50	
31	4-OHE_2-2-Cys	0.13	
32	4-OHE_1-2-Cys	0.06	
33	4-OHE_2-2-NAcCys	0.29	
34	4-OHE_1-2-NAcCys	0.28	
35	4-OHE_2-1-N7Gua	0.48	2.81
36	4-OHE_1-1-N7Gua	2.33	
37	4-OHE_2-1-N3Ade	137.78	137.90
38	4-OHE_1-1-N3Ade	0.13	
39	2-OHE_2-6-N3Ade	0.06	0.07
40	2-OHE_1-6-N3Ade	0.02	
(Ratio-4)[2] × 1,000			935
(Ratio-2)[3] × 1,000			1
(Ratio-4) + (Ratio-2) × 1,000			936

[1]Typically, each 2-ml urine sample was analyzed at least 2 times. The data obtained from LC/MS-MS were processed and normalized to creatinine levels. Since the E_1 and E_2 derivatives are interconvertible, the total amount for each E_1 plus E_2 derivative in the various categories are presented in the last column and used for calculating the final ratios of depurinating adducts to the respective metabolites and conjugates.

[2] $$\frac{4-\text{OHE}_1(\text{E}_2)-1-\text{N3Ade}+4-\text{OHE}_1(\text{E}_2)-1-\text{N7Gua}}{4-\text{catechol estrogens}+4-\text{catechol estrogen conjugates}} = \frac{\text{No. 37 + 38 + 35 + 36}}{\text{No. 8 + 9 + 14 + 15 + 29 through 34}}$$

[3] $$\frac{\text{2 - OHE1 (E2) - 6 - N3Ade}}{2-\text{catechol estrogens}+2-\text{catechol estrogen conjugates}} = \frac{\text{No. 39 + 40}}{\text{No. 6 + 7 + 12 + 13 + 16 through 28}}$$

[4]Free E_2 and E_1 in the urine sample.
[5]All 2-$OHE_1(E_2)$ conjugates.
[6]All 4-$OHE_1(E_2)$ conjugates.

TABLE III – URINARY LEVELS OF ESTROGEN COMPOUNDS IN HEALTHY WOMEN, HIGH-RISK WOMEN AND WOMEN WITH BREAST CANCER

No.	Compound	Control (n = 46)		High Risk (n = 12)			Breast Cancer (n = 18)		
		Median	Min–Max	Median	Min–Max	p-value[3]	Median	Min–Max	p-value[4]
1	Androstenedione	9.9	2.1–108	4.2	1.3–11.5	0.003[3]	5.5	0.4–95.1	0.047
2	Testosterone	2.2	0.2–16.5	0.8	0.2–2.8	**0.008**[5]	1.1	0.5–3.7	0.050
3	E_1-Sulfate	5.0	0.1–382	2.4	0.1–10.6	**0.087**	1.1	0.1–121	0.032
4	E_2	31.7	9.1–3865	11.4	4.7–80.0	**0.007**	28.1	3.6–151	0.943
5	E_1								
6	2-OHE_2	10.4	1.7–564	7.3	1.6–26.5	0.035	5.6	0.0–38.8	**0.006**
7	2-OHE_1								
8	4-OHE_2	12.4	2.4–157	8.1	2.9–43.3	0.138	5.2	0.0–28.0	**0.008**
9	4-OHE_1								
10	16α-OHE_2	168	10.3–638	33.7	0.0–279	**0.001**	10.9	0.0–86.3	**<0.001**
11	16α-OHE_1								
12	2-OCH_3E_2	49.7	4.7–568	31.1	6.5–452	0.275	26.8	2.2–171	0.044
13	2-OCH_3E_1								
14	4-OCH_3E_2	73.1	12.6–3979	5.9	1.6–37.1	**<0.001**	21.5	4.5–53.2	**<0.001**
15	4-OCH_3E_1								
16	2-OH-3-OCH_3E_2								
17	2-OH-3-OCH_3E_1								
18	2-OHE_2-1-SG	11.2[1]	0.8–79.8[1]	4.6[1]	1.2–18.7[1]	**0.005**[1]	3.1[1]	1.1–16.9[1]	**0.001**[1]
19	2-OHE_2-4-SG								
20	2-OHE_1-1-SG								
21	2-OHE_1-4-SG								
22	2-OHE_2-1+4-Cys								
23	2-OHE_1-1-Cys								
24	2-OHE_1-4-Cys								
25	2-OHE_2-1-NAcCys								
26	2-OHE_2-4-NAcCys								
27	2-OHE_1-1-NAcCys								
28	2-OHE_1-4-NAcCys								
29	4-OHE_2-2-SG	2.7[2]	0.7–24.6[2]	1.4[2]	0.6–6.8[2]	0.032[2]	1.3[2]	0.4–8.9[2]	0.027[2]
30	4-OHE_1-2-SG								
31	4-OHE_2-2-Cys								
32	4-OHE_1-2-Cys								
33	4-OHE_2-2-NAcCys								
34	4-OHE_1-2-NAcCys								
35	4-OHE_2-1-N7Gua	0.7	0.0–4.8	1.2	0.2–2106	0.238	1.6	0.4–11.8	**0.007**
36	4-OHE_1-1-N7Gua								
37	4-OHE_2-1-N3Ade	0.7	0.0–18.8	1.8	0.5–138	**0.007**	1.2	0.1–288	0.085
38	4-OHE_1-1-N3Ade								
39	2-OHE_2-6-N3Ade	0.1	0.0–6.5	0.1	0.0–0.7	0.999	0.1	0.0–5.4	0.960
40	2-OHE_1-6-N3Ade								

[1]All 2-$OHE_1(E_2)$ conjugates.–[2]All 4-$OHE_1(E_2)$ conjugates.–[3]Bonferroni-adjusted p-value for comparing control *versus* high risk using Mann–Whitney test.–[4]Bonferroni-adjusted p-value for comparing control *versus* breast cancer by using Mann–Whitney test.–[5]Significant p-values are shown in bold.

risk participants, and the median 4-OHE_2-1-N3Ade/4-OHE_1-1-N3Ade values were lower for controls compared to high risk participants. Compared to breast cancer participants, the median 2-OHE_2/2-OHE_1, 4-OHE_2/4-OHE_1, 16α-OHE_2/16α-OHE_1, 4-OCH_3E_2/4-OCH_3E_1, 2-$OHE_1(E_2)$ GSH conjugate and derivative values were higher for controls, while the median 4-OHE_2-1-N7Gua/4-OHE_1-1-N7Gua values were lower for controls. Of particular interest are the significantly lower levels of the methoxycatechol estrogens in the women with breast cancer or at high risk compared to the control women, because this represents a major protective pathway in estrogen metabolism. In addition, the levels of the 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua adducts are higher in the women with breast cancer or at high risk than in the control women, although only 2 of the differences are statistically significant.

Depurinating estrogen-DNA adducts in the 3 groups of women

In the second analysis, the ratios of depurinating N3Ade and N7Gua adducts to the sum of estrogen metabolites and conjugates in urine samples from healthy control women are generally low (Fig. 4). In contrast, high ratios of these adducts to estrogen metabolites and conjugates were observed in urine from high-risk women (Gail Model score >1.66%) and women with breast carcinoma. In general, the value obtained from the high-risk women and women with breast carcinoma derives from the ratio between a high level of adducts and low levels of metabolites and conjugates. In some women, however, the level of adducts was not particularly high, but the levels of metabolites and conjugates were very low, suggesting that a substantial proportion of the metabolites was converted to adducts.

In the sum of the ratios of depurinating adducts to estrogen metabolites and conjugates, the preponderant role is played by the N3Ade and N7Gua adducts of 4-$OHE_1(E_2)$, whereas the adducts of 2-$OHE_1(E_2)$ play a very minor role. For example, for the high-risk subject presented in Table II, the overall adduct ratio is 936, but the contribution of 2-$OHE_1(E_2)$-6-N3Ade is 1, whereas the contribution of 4-$OHE_1(E_2)$-1-N3Ade plus 4-$OHE_1(E_2)$-1-N7Gua is 935. In general, the average contribution of the 2-$OHE_1(E_2)$-6-N3Ade adducts is ~2.5% of the total, whereas the predominant contribution of ~97.5% derives from the 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua adducts. The observation of high levels of depurinating estrogen-DNA adducts in urine from high-risk women, as well as subjects with breast carcinoma (Fig. 4), is consistent with the hypothesis that these adducts are a causative factor in the etiology of breast cancer.

Analysis by subject characteristics

We first analyzed the data using the ratio of depurinating N3Ade and N7Gua adducts to the sum of their respective estrogen

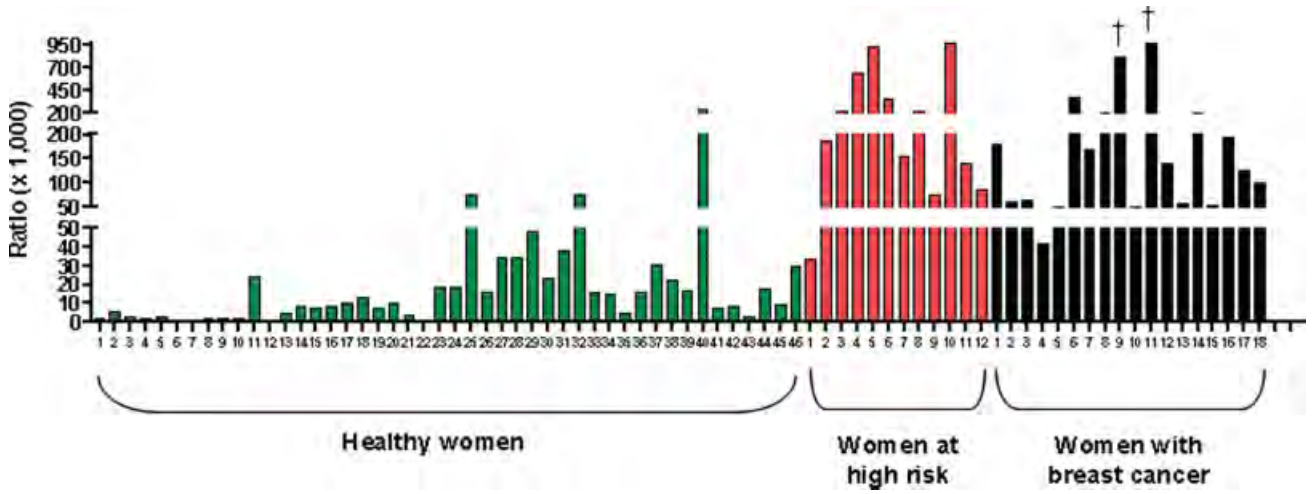

FIGURE 4 – Depurinating estrogen-DNA adducts in the urine of healthy women, high-risk women and women with breast cancer. The ordinate of this bar graph corresponds to the ratio of depurinating DNA adducts divided by their respective estrogen metabolites and conjugates:

$$\left(\frac{4-\mathrm{OHE_1(E_2)}-1-\mathrm{N3Ade}+4-\mathrm{OHE_1(E2)}-1-\mathrm{N7Gua}}{4-\text{catechol estrogens}+4-\text{catechol estrogen conjugates}}+\frac{2-\mathrm{OHE_1(E_2)}-6-\mathrm{N3Ade}}{2-\text{catechol estrogens}+2-\text{catechol estrogen conjugates}}\right)\times 1000$$

The mean sum of the ratios for control women was significantly lower than those for the high-risk women ($p < 0.001$) and women with breast cancer ($p < 0.001$). The mean sums of the ratios for high-risk women and women with breast cancer were not significantly different ($p = 0.62$). †These are 2 urine samples from the same subject, collected 11 weeks apart. Statistical calculations used 1 average value for this subject.

TABLE IV – SUBJECT CHARACTERISTICS

Characteristic	Health status		
	Healthy ($n = 37$)	High risk ($n = 12$)	Breast cancer ($n = 7$)
Age at recruitment in years, mean (SD)	49 (7.85)	52 (6.09)	57 (12.16)
Age at menarche in years, mean (SD)	12 (1.45)	12 (1.44)	13 (1.25)
Menopausal Status, *n* (%)			
Premenopausal	17 (46%)	6 (50%)	3 (43%)
Postmenopausal	20 (54%)	6 (50%)	4 (57%)
Parity			
0	6 (16%)	0 (0%)	0 (0%)
1	3 (8%)	0 (0%)	0 (0%)
2	13 (35%)	7 (58%)	4 (57%)
3	11 (30%)	3 (25%)	0 (0%)
≥4	4 (11%)	2 (17%)	3 (43%)

TABLE V – RESULTS OF UNIVARIATE MULTIVARIATE LINEAR REGRESSION OF RATIO

Covariate	Univariate regression		Multivariate regression	
	Regression coefficient	*p*-value	Regression coefficient	*p*-value
Health status	103.60	0.005	108.56	0.007
Postmenopausal	35.66	0.51	41.18	0.44
Parity	15.01	0.42	−7.29	0.71

metabolites and conjugates in urine samples as a continuous variable. Analysis using one-way ANOVA revealed a significant difference among the groups ($p < 0.001$). Additional *post hoc* analysis using a Bonferroni correction for multiple comparisons revealed significantly higher means for high risk subjects [mean 336.45, standard deviation (SD) 331.92] compared to controls (mean 20.51, SD 37.01, $p < 0.001$) and for breast cancer patients (mean 176.28, SD 205.68, $p < 0.001$). The mean for patients known to be at high risk was not significantly different from that of the breast cancer group ($p = 0.62$).

A limitation of the study is that most of the group of healthy women (42 of 46) were Italian, whereas the remaining healthy women, high-risk women and women with breast cancer were American. All of the subjects in our study, however, were Caucasian. The 3 groups (healthy, high-risk and breast cancer) had similar mean age at recruitment, mean age at menarche and menopausal status (Table IV). These similarities in subject characteristics support the validity of comparing the ratios of adducts to their respective metabolites and conjugates in these 3 groups of women.

Subject characteristics of age at recruitment, age at menarche, menopausal status, and parity were available for 56 of the 75 subjects (Table IV). The mean age of our entirely Caucasian sample was 50 years (SD 8.5). The average age at menarche was 12.0 years (SD 1.4). Only 11% of the women were nulliparous and 43% had at least 2 children. Twenty-six (46%) women were premenopausal at recruitment, 30 (54%) were postmenopausal (they did not have menstrual cycles in the last 12 months before recruitment). Analysis using one way ANOVA revealed that health status, that is breast cancer cases *versus* high risk and healthy individuals, was significantly associated with age at recruitment ($p = 0.048$). Specifically, the mean age (years) at recruitment for healthy women was 49 (SD 7.8), 52 (SD 6.1) for women at high risk and 57 (SD 12.2) for breast cancer cases. Age at menarche was not statistically different across the disease status groups ($p = 0.534$). Analysis using a χ^2 test did not reveal an association between health status and menopausal status ($p = 0.95$) or parity (parous *vs*. nulliparous) ($p = 0.15$).

The correlation coefficient was used to examine the association between the ratio and subject characteristics. We observed evidence of significant correlation between parity and ratio ($r = 0.36$, $p = 0.007$) and marginally significant correlation between the ratio and menopausal status ($r = 0.26$, $p = 0.06$). Age at recruitment and age at menarche were not significantly associated with the ratio.

Linear regression was used to assess the association between disease status and ratio adjusted for age at recruitment, age at menarche, menopausal status (categorical) and parity for the 56

subjects with patient characteristics available (Table V). After accounting for these characteristics, the ratio was significantly associated with health status. Specifically, the multivariate coefficient for disease status (108.6) was statistically significant ($p = 0.007$) in a model that explained 10% ($p = 0.040$) of variance in the ratio after accounting for covariates. All other covariates did not reach the usual level of significance of 0.05 (Table V).

Interpretation of results

The observation of high ratios of depurinating estrogen-DNA adducts to their corresponding metabolites and conjugates in urine samples from both high-risk women and women with breast cancer supports the hypothesis that formation of estrogen-DNA adducts is the first critical step in the initiation of breast cancer.[1] In addition, these results suggest that this assay may provide a diagnostic tool for early detection of breast cancer risk. At this point, we do not know how far in advance this assay would predict the development of a detectable tumor. Further studies are required to address this question.

In addition, we can hypothesize that the ratio of depurinating estrogen-DNA adducts to their metabolites and conjugates can be used to monitor the efficacy of putative preventive compounds in balancing estrogen activation and deactivation. Minimizing formation of catechol estrogen quinones and/or their reaction with DNA should reduce the risk of developing breast cancer.

Conclusions

UPLC/MS-MS can be used to analyze depurinating estrogen-DNA adducts, estrogen metabolites and estrogen conjugates in 2-ml urine specimens. The ratio of adducts to their corresponding metabolites and conjugates provides a biomarker that can be used to distinguish women known to be at high risk of developing breast cancer (Gail Model score >1.66%) and those with breast cancer from healthy control women. The development of such biomarkers could be invaluable in assessing breast cancer risk and response to preventive treatment.

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MOLECULAR BIOLOGY CORE – SUTTER

A. INTRODUCTION

The molecular biology core of this BCCOE brings high throughput molecular analyses to the specific aims of this center. The primary capacity of this core is high throughput gene expression analysis facilitated by Affymetrix GeneChip technology. In addition to massively parallel analysis of gene expression, new technologies, released in 2005, permitted the expansion of these facilities into high resolution analysis of chromosome structure. As determined at the December 6, 2004 meeting of the BCCOE held in Washington, DC, the efforts of this core was focused on Specific Aim 2 (Russo) of the center: to determine the effects of estrogen and its metabolites on the progressive steps of neoplastic transformation of human breast epithelial cells (HBECs) and to determine whether the neoplastic phenotypes and genotypes thus induced can be abrogated by known and new preventive agents. In addition, Dr. Sutter continued his seminal work on CYP1B1, the enzyme responsible for production of the 4-hydroxyestradiol metabolite.

B. BODY

B-i. Methods and procedures

B.i.a. Cell Lines, DNA and RNA

The cell lines and the malignant transformation protocol are shown in Fig. 1 and described previously. In this work, three individual samples of each cell line were analyzed as independent replicates. For comparison to the previous work we report the current and (previous) cell sample designations: MCF10F samples 1,2 3 (MCF-10F 1,2,3); trMCF samples 1,2 3 (E_2-70 nM 1,2,3); bsMCF (C5 1,2,3) and caMCF (L1, L4, L8). The designation E_2-70 nM referred to treatment conditions resulting in cell transformation; C5 referred to the position of the well in the selection chamber; L1, L4 and L8 referred to the cell line derived from the tumor of C5 cells in animal 1 (L1) and so forth. For isolation of the bcMCF cell lines, bsMCF cells were plated at low density and observed under the microscope. Individual colonies were isolated using cloning rings, selective trypsinization and plating, giving rise to six clones designated Clones A, B, C, F, H, and I. bcMCF clones A, B and F were analyzed in this study. Furthermore, the Clone A cell line of bcMCF was tested for its tumorigenic capacity in 45 day old female SCID mice, which were obtained from the FCCC animal care facility, as previously described. Animals were housed four to a cage and maintained in a laminar flow rack at 72 ^{0}F with a 12h light/dark cycle. They received water and food ad libitium. The cells were injected into the mammary fat pad of the abdominal region of the mice at a concentration of 10-15 x 10^6 cells suspended in 0.1 ml of sterile phosphate buffered saline. The animals were palpated twice a week for detection of tumor development and were followed for up to six months post injection. Animals were killed by carbon dioxide inhalation. Each animal was autopsied, carefully examined for identification of visceral metastasis and palpable tumors were dissected from the skin (1). High molecular weight genomic DNA and total cellular RNA were isolated from three individual samples of each cell line: MCF10F, trMCF, bsMCF, bcMCF, and caMCF. For DNA, cells were treated with lysis buffer containing 20 mM Tris-CL, pH 8.0, 100 mM NaCl, 25 mM EDTA, pH 8.0, 0.5% sodium dodecyl sulphate, and 200 μg/mL proteinase K for 15 min at 65 ^{o}C

with gentle agitation. The samples were cooled on ice and treated with 100 μg/mL RNase A at 37 ^{o}C for 30 min. The samples were extracted once with buffered phenol, and again with chloroform:isoamyl alcohol (24:1). The aqueous layer was adjusted to 0.75 M ammonium acetate and the DNA was precipitated by the addition of 2.5 volumes of 100% ethanol. The precipitate was washed with 70% ethanol, dried and dissolved in sterile water. Total cellular RNA was isolated using the TRIZOL (Life Technologies, Gaithersburg, MD) modification of the guanidinium thiocyanate procedure. The concentration and quality of the DNA and RNA was determined spectrophotometrically and by capillary gel electrophoresis (Agilent 2100 Bioanalyzer, Palo Alto, CA).

B-i-b. Genotyping and microarray assays

ffymetrix 100k Single Nucleotide Polymorphism (SNP) mapping was performed using the combined Xba I (Mapping50K_Xba240) Hind III (Mapping50K_Hind240) GeneChip mapping Array set according to the manufacturer's recommended procedures (Affymetrix, Santa Clara, CA) with the following modifications. The time of the restriction endonuclease digestions was increased to 6 hr at 37 ^{o}C, and the ligation reaction was carried out overnight at 16 ^{o}C. Using this 100k SNP set, one obtains allele information at a mean intermarker distance of 23.6 kb and median intermarker distance of 8.5 kb. The average heterozygosity of each SNP is 0.30. Gene expression microarray analysis was performed using the Affymetrix HG-U133_Plus_2 Array, measuring more than 47,000 transcripts. Eight μg of total RNA was used in the cDNA synthesis reaction. After hybridization, the chips were washed and scanned on the GeneChip Scanner 3000 (Affymetrix). The genotype calls (heterozygous or homozygous) were determined using the Affymetrix GTYPE v4.0; the P(Present)- or A(Absent)-calls of the probes in the gene expression chips were determined using the Affymetrix GCOS v1.4. The microarray data have been submitted to the National Center for Biotechnology Information (NCBI)'s Gene Expression Omnibus repository (series accession no. GSE5116.

B-i-c. Data analysis

The chromosome copy number changes and LOH were determined using dChip (2). The .CEL files of Mapping50K_Hind240 and Mapping50K_Xba240 chips and their corresponding .TXT files containing the SNP genotype calls were put into dChip to calculate the intensities of probes. The human genome release v17 was used to provide the genome information files (refGene and cytoBand files) that were used for the SNP data analysis in dChip. The output files containing the SNP intensities and SNP genotype calls were merged together for the 100k SNP analysis. The MCF10F cell line served as diploid reference for detection of copy number changes. The genotype of the MCF10F cell line was also interrogated using the Affymetrix CNAT v3.0, in order to detect potential aneuploidy. Genomic smoothed analysis (GSA) with 0.5Mbp distance was used to delineate the copy number change and LOH. The intensities of probe sets in the HG-U133_Plus_2 Genechips were calculated by dChip software using the Perfect-match/Mismatch difference model after invariant-set normalization. A gene is considered expressed in the group of interest if the gene is "Present" in all 3 samples of that group. Differentially expressed genes were identified by pairwise comparison using MCF10F as the reference. The significance level was $p < 0.05$ in an unpaired t-test of the log transformed expression values. To identify and extract the expression data for probesets corresponding to

genes located within specific regions of individual chromosomes, the Entrez Gene ID and corresponding location in Mb were used to search the annotation file of the HG-U133_Plus_2 Genechip. To integrate the Entrez Gene ID and associated gene with the human genome map, we used the GeneLoc tool of the Weismann Institute of Science.

B-i-d. Immunohistochemistry

The purified serum IgG was used at a concentration of either 10 μg/mL (CYP19) or 10 μg/mL (CYP1B1). Non-specific staining was assessed using normal rabbit serum (Sigma) at a dilution of 1:5000. For the tissue microarray analysis, paraffin-embeded human breast cancer (invasive ductal carcinoma) IMH-36460 samples for cancer metastasis and normal breast tissue were obtained as tissue microarrays from Imgenex (San Diego, CA, USA). The slides were deparaffinized in xylene (twice for 4 min) followed by washing in 100% ethanol (4 min), 95% ethanol (2 min), 80% ethanol (15 s) and distilled water as described previously (3) using diaminobenzidine as the chromagen. The slides were washed with distilled water for 1 min and counterstained for 1 min with hematoxilin.

B-i-e. Enzyme kinetics assays of recombinant human CYP1B1

The recombinant human CYP1B1 protein was expressed in S. cerevisiae as described previously (4). The microsome preparation was evaluated by measuring the enzymatic activity as described before (5). The inhibition kinetics of CYP1B1 was determined in a range expected to produce 30 to 90 percent inhibition. A fixed substrate concentration and varying inhibitor concentrations were used. An IC_{50} value was determined at the point where 50% inhibition of the enzyme's catalytic activity occurred. The E2 hydroxylation assay was performed with the addition of inhibitor and has been previously described in detail (5). Inhibition was calculated as percent of product formation compared to the corresponding control (enzyme-substrate reaction) without the inhibitors.

B-ii. Results

B-ii-a. Malignant Cell Transformation by Estogens

Epithelial to Mesenchymal Transition in Human Epithelial Cells Transformed by 17-beta-estradiol.

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Abstract

The estrogen-dependence of breast cancer has long been recognized, however, the role of 17β-estradiol (E2) in cancer initiation was not known until we demonstrated that it induces complete neoplastic transformation of the human breast epithelial cells MCF-10F. E2-treatment of MCF-10F cells progressively induced high colony efficiency and loss of ductulogenesis in early transformed (trMCF) cells, invasiveness in a Matrigel invasion chamber (bsMCF and bcMCF),

and carcinoma formation in SCID mice (caMCF). These phenotypes correlated with gene dysregulation during the progression of the transformation phenomenon. The highest number of dysregulated genes was observed in caMCF cells, being slightly lower in bcMCFcells, and lowest in trMCF cells. This order was consistent with the extent of chromosome aberrations (caMCF > bcMCF >> > trMCF). Chromosomal amplifications were found in 1p36.12-pter, 5q21.1-qter and 13q21.31-qter. Losses of the complete chromosome 4 and of 8p11.21-23.1 were found only in tumorigenic cells. In tumor-derived cell lines, additional losses were found in 3p12.1-14.1, 9p22.1-pter and 18q11.21-qter. Functional profiling of dysregulated genes revealed progressive changes in the integrin signaling pathway, inhibition of apoptosis, acquisition of tumorigenic cell surface markers and epithelial to mesenchymal transition. In both, bcMCF and caMCF cells, the levels of E-cadherin, EMA, and various keratins were low and CD44E/CD24 were negative, whereas SNAI2 (CDH1 repressor), vimentin, S100A4, FN1, HRAS and TGFβ1, and CD44H were high. The phenotypic and genomic changes triggered by estrogen exposure that lead normal cells to tumorigenesis confirm the role of this steroid hormone in cancer initiation.

Progressive loss of CST6 expression in a model of malignant cell transformation of human breast epithelial cells.

Sandra V. Fernandez[1], Yong Huang[2], Irma H Russo[1], Thomas R. Sutter[2], and Jose Russo[1].

Abstract

Cystatin E/M (CST6) is a potent inhibitor of endogenous mammalian lysosomal cysteine proteases, including cathepsin B and cathepsin L which promote tumor growth, invasion and metastasis through degradation of extracellular connective matrices and endothelial cell growth-directed activities. In the present work we demonstrate that CST6 is silenced by methylation during the early stages of 17 ˜ estradiol neoplastic transformation of the human breast epithelial cells MCF10F. In the in vitro-in vivo model of breast cancer induced by estrogen, different stages in the breast tumor progression are represented being the MCF-10F, the normal stage; trMCF, transformed but non tumorigenic; bsMCF, the invasive stage and tumorigenic in heterologous host ; and the more advanced tumorigenic stage represented by caMCF cells . Levels of cystatin E/M (CST6) mRNA were down-regulated in trMCF, bsMCF and caMCF cells. The invasive bsMCF cells have very low expression of CST6 and higher levels of expression of cathepsin C and cathepsin L-like 3, whereas the caMCF cells showed low levels of CST6 and increase in cathepsin C, cathepsin B and cathepsin L. We show that 5-aza-dC but not trichostatin was able to increase CST6 expression in MCF-10F, trMCF and bsMCF cells, indicating that CST6 expression is regulated, in part, by DNA methylation. Epigenetic control of this gene appears as an early event in the process of estrogen-induced malignant cell transformation of human MCF-10F cells.

Work in Progress
Russo/Sutter labs

Studies in Dr. Russo's laboratory demonstrated that treatment of transformed and matrigel selected cell with hCG could reverse or inhibit the phenotypes of cell transformation and invasion. Microarray analysis performed in the Sutter lab has revealed underlying gene

expression mechanisms that correspond to these observed phenotypes. This important collaboration is the first work to understand molecular mechanisms for chemoprevention of malignant cell transformation mediated by estrogen.

B-ii-b. 17-beta-estradiol metabolism and Cytochrome P4501B1

Regioselective 2-hydroxylation of 17β-estradiol by rat cytochrome P4501B1
Mostafizur Rahman[a,b], Carrie Hayes Sutter[a], Gary L. Emmert[b] and Thomas R. Sutter[a,b*]

Abstract

Previous work demonstrated that human cytochrome P4501B1 (CYP1B1) forms predominantly 4-hydroxyestradiol (4-OHE2), a metabolite which is carcinogenic in animal models. Here, we present results from kinetic studies characterizing the formation of 4-OHE2 and 2-hydroxyestradiol (2-OHE2) by rat CYP1B1 using 17β-estradiol (E2) as a substrate. K_m and K_{cat} values were estimated using the Michaelis-Menten equation. For rat CYP1B1, the apparent K_m values for the formation of 4-OHE2 and 2-OHE2 were 0.61 ± 0.23 and 1.84 ± 0.73 μM; the turnover numbers (K_{cat}) were 0.23 ± 0.02 and 0.46 ± 0.05 pmol/min/pmol P450; and the catalytic efficiencies (K_{cat}/K_m) were 0.37 and 0.25, respectively. For human CYP1B1, the apparent K_m values for the formation of 4-OHE2 and 2-OHE2 were 1.22 ± 0.25 and 1.10 ± 0.26; the turnover numbers were 1.23 ± 0.06 and 0.33 ± 0.02; and the catalytic efficiencies were 1.0 and 0.30, respectively. The turnover number ratio of 4- to 2-hydroxylation was 3.7 for human CYP1B1 and 0.5 for rat CYP1B1. These results indicate that although rat CYP1B1 is a low K_m E2 hydroxylase, its product ratio, unlike the human enzyme, favors 2-hydroxylation. The K_i values of the inhibitor 2,4,3′,5′-tetramethoxystilbene (TMS) for E2 4- and 2-hydroxylation by rat CYP1B1 were 0.69 and 0.78 μM, respectively. The K_i values of 7,8-benzoflavone (α-NF) for E2 4- and 2-hydroxylation by rat CYP1B1 were 0.01 and 0.02 μM, respectively. The knowledge gained from this study will support the rational design of CYP1B1 inhibitors and clarify results of CYP1B1 related carcinogenesis studies performed in rats.

Co-expression of CYP19 and CYP1B1 in tumor epithelial cells of invasive ductal carcinoma
Mostafizur Rahman [1, 2, 3], Sigurd F. Lax [4], Carrie Hayes Sutter [1, 3], Gary L. Emmert [2], Jose Russo [5], William R. Miller [6], Richard J. Santen [7], and Thomas R. Sutter [1, 2, 3, *]

Abstract

A cytochrome P45019 (CYP19, aromatase) polypeptide expressed as a hexahistidine-tagged fusion protein in E coli was purified and used to produce a polyclonal antibody in rabbit. Immunoblot analysis showed that this antibody was very specific and capable of detecting human CYP19 protein. Immunohistochemical analyses of CYP19 and CYP1B1 in a panel of 29 cases of invasive ductal carcinoma of the breast, showed epithelial cell staining for CYP19 in 76% of samples and for Cyp1B1 in 97%. Since CYP1B1 and CYP19 were co-expressed in breast cancer and since both enzymes are efficient estrogen hydroxylases, we investigated whether CYP1B1 expression may affect the disposition of Aromatase inhibitors (AIs). To do this, we determined the inhibition properties against CYP1B1-catalyzed hydroxylation of 17β-

estradiol (E_2) by a series of AIs including three steroidal inhibitors; formestane, exemestane, and androstenedione, and five non-steroidal inhibitors; aminoglutethimide, fadrozole, anastrozole, letrozole, and vorozole. Of the eight compounds tested, only vorozole exhibited inhibition of CYP1B1 activity with IC_{50} values of 17 and 21 μM for 4-OHE_2 and 2-hydroxyestradiol (2-OHE_2), respectively. The estimated K_i values of vorozole for E_2 4- and 2-hydroxylation were 7.26 and 6.84 μM, respectively. Spectrophotometric studies showed that vorazole is a type II inhibitior of CYP1B1. None of the other seven AIs tested showed significant inhibition towards CYP1B1 activity and the IC_{50} values for 4-OHE_2 and 2-OHE_2 were all greater than 100 μM. This study shows that with the exception of vorozole, the aromatase inhibitors are selective for CYP19 relative to CYP1B1. Thus, although both CYP19 and CYP1B1 are expressed in a high percentage of breast cancers, CYP1B1 does not appear to be an important determinant of the disposition or metabolism of the aromatase inhibitors tested.

Genetic variation of Cytochrome P450 1B1 (CYP1B1) and risk of breast cancer among Polish women.
Gaudet MM, Chanock S, Lissowska J, Berndt SI, Yang XR, Peplonska B, Brinton LA, Welch R, Yeager M, Bardin-Mikolajczak A, Sherman ME, Sutter TR, Garcia-Closas M.

Abstract

Four single nucleotide polymorphisms (SNPs) in CYP1B1 (Ex2 + 143 C > G, Ex2 +356 G > T, Ex3 + 251 G > C, Ex3 + 315 A > G) cause amino acid changes (R48G, A119S, L432V and N453S, respectively) and are associated with increased formation of catechol estrogens; however, epidemiologic evidence only weakly supports an association between these variants and breast cancer risk. Because genetic variability conferring increased susceptibility could exist beyond these putative functional variants, we comprehensively examined the common genetic variability within CYP1B1. A total of eight haplotype-tagging (ht)SNPs (including Ex3 + 315 A > G), in addition to two putatively functional SNPs (Ex2 + 143 C > G and Ex3 + 251 G > C), were selected and genotyped in a large case-control study of Polish women (1995 cases and 2296 controls). Haplotypes were estimated using the expectation-maximization algorithm, and overall differences in the haplotype distribution between cases and controls were assessed using a global score test. We also evaluated levels of tumor CYP1B1 protein expression in a subset of 841 cases by immunohistochemistry, and their association with genetic variants. In the Polish population, we observed two linkage disequilibrium (LD)-defined blocks. Neither haplotypes (global P-value of 0.99 and 0.67 for each block of LD, respectively), nor individual SNPs (including three putatively functional SNPs) were associated with breast cancer risk. CYP1B1 was expressed in most tumor tissues (98%), and the level of expression was not related to the studied genetic variants. We found little evidence for modification of the estimated effect of haplotypes or individual SNPs by age, family history of breast cancer, or tumor hormone receptor status.
The present study provides strong evidence against the existence of a substantial overall association between common genetic variation in CYP1B1 and breast cancer risk.

Hormonal markers in breast cancer: co-expression, relationship with pathologic characteristics and risk factor associations in a population-based study
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Abstract

The objective of this study was to evaluate co-expression patterns of markers related to hormone pathways in breast cancer tissue and their relationship with pathologic characteristics and risk factors. We evaluated immunohistochemical expression of 17 markers in tissue microarrays prepared from 842 invasive breast carcinomas collected in a population-based case-control study conducted in Poland. Based on marker correlations, factor analysis identified four major co-expression patterns (factors): the "nuclear receptor factor" (ER-α, PR, androgen receptor, cyclin D1, and aromatase); the "estrogen metabolism / ER-β factor" (ER-β, peroxisome proliferators-activated receptor, steroid sulfatase, estrogen sulfonotransferase , and cytochrome P450 1B1; the "HER-2 factor" (HER-2, E-cadherin, cyclooxygenase-2, aromatase, STS; and the "proliferation factor" (cytokeratin 5, cytokeratin 5/6, EGFR, P53). In contrast with other factors, the estrogen metabolism / ER-β factor did not correspond to previously defined molecular subtypes by global gene expression studies. High scores for this factor were associated with high tumor grade (P-heterogeneity =0.02), younger age at menarche (P-heterogeneity = 0.04), lower current BMI among premenopausal women (P-heterogeneity = 0.01), and older age at menopause (P-heterogeneity = 0.04). High scores for the proliferation factor were also associated with early menarche (P-heterogeneity < 0.0001), and in contrast to the estrogen metabolism / ER-β factor, higher current BMI among premenopausal women (P-heterogeneity = 0.03). Our results suggest that analysis of biologically relevant markers may reveal associations with pathologic characteristics and risk factors that overlap with and add to relationships discovered by analysis of molecular subtypes defined by expression profiling.

D. REPORTABLE OUTCOMES

D-i. in press

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D-ii. submitted

4. Yong Huang, [1] Sandra Fernandez, [2] Shirlean Goodwin, [1] Patricia A. Russo, [2] Irma Russo, [2] Thomas R. Sutter, [1] and Jose Russo [2, *] 2007. Epithelial to Mesenchymal Transition in Human Epithelial Cells Transformed by 17-beta-estradiol. Submitted

5. Sandra V. Fernandez[1], Yong Huang[2], Irma H Russo[1], Thomas R. Sutter[2], and Jose Russo[1]. 2007. Progressive loss of CST6 expression in a model of malignant cell transformation of human breast epithelial cells. Submitted

D-ii. in preparation

6. Mostafizur Rahman [1, 2, 3], Sigurd F. Lax [4], Carrie Hayes Sutter [1, 3], Gary L. Emmert [2], Jose Russo [5], William R. Miller [6], Richard J. Santen [7], and Thomas R. Sutter [1, 2, 3,] 2007. Co-expression of CYP19 and CYP1B1 in tumor epithelial cells of invasive ductal carcinoma.

7. Xiaohong R. Yang(1), Ruth M. Pfeiffer(1), Montserrat Garcia-Closas(1), David L.Rimm(2), Jolanta Lissowska(3), Louise A.Brinton(1), Beata Peplonska(4), Stephen M.Hewitt(5), Richard Cartun(6), Daniza Mandich(6), Hiro Sasano(7), Dean B. Evans(8), Thomas R. Sutter(9), Mark E.Sherman(1) 2007. Hormonal markers in breast cancer: co-expression, relationship with pathologic characteristics and risk factor associations in a population-based study

E. CONCLUSIONS

We have continued to elaborate on our important findings of the role of CYP1B1 in estrogen hydroxylation and the risk of developing breast cancer. During this period, we have made contributions to the areas of comparative metabolism, aromatase inhibition, genetic predisposition and tumor classification. In our genomic analysis of the cell transformation model established by the Russo laboratory, we identified important molecular events leading to the expression of tumorigenic markers and epithelial-mesenchymal transition (EMT). EMT is an important cellular determinant of invasiveness and metastasis. By identifying these characteristics occurring during malignant cell transformation by estrogen in the ER-negative cell line MCF-10F, it identifies a new and essential cell model for understanding the especially aggressive characteristics of ER-negative tumors.

F. REFERENCES

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ANALYTICAL CORE – ROGAN

A. Introduction

The Analytical Core provides consistent analytical power to the research projects so that estrogen metabolites, estrogen conjugates and estrogen-DNA adducts can be identified and quantified with the most sensitivity and reliability. The HPLC with multi-channel electrochemical detection enables detection of 34 metabolites and conjugates at the picomole level in one run. A second set-up is used exclusively to analyze the 6 depurinating catechol estrogen-DNA adducts, which need a different elution buffer to achieve separation. Our newer ultraperformance liquid chromatography/tandem mass spectrometry (UPLC/MS/MS) instrumentation has increased the sensitivity of our analyses to the femtomole level and provides confirmation of structures.

B. Body

B-i. Methods and Procedures

The Analytical Core uses both HPLC with electrochemical detection and UPLC/MS/MS to analyze estrogen metabolites, conjugates and depurinating DNA adducts. A variety of types of samples are analyzed. These include *in vitro* reaction mixtures, human and animal tissue extracts, cell culture medium extracts, and human fluids, such as urine, serum and nipple aspirate fluid. Depending on the sample, preparation for analysis is as simple as filtration through a 5,000-molecular weight filter, or as complex as grinding minced tissue in liquid nitrogen, incubation with glucuronidase/sulfatase, passage through a Sep-Pak column to extract desired analytes, and filtration through a 5,000-molecular weight filter.

B-ii. Results

In the past year we have used our Acquity UPLC (Waters, Inc), which is coupled with a MicroMass QuattroMicro tandem mass spectrometer, to separate and analyze 34 estrogen metabolites and GSH conjugates [and their breakdown products containing cysteine (Cys) and *N*-acetylcysteine (NAcCys)] and 6 depurinating DNA adducts by UPLC/MS/MS.

In the past year we primarily analyzed human samples, including nipple aspirate fluid from women with and without breast cancer, urine from women with and without breast cancer and at high risk of breast cancer [1], and serum from women without breast cancer. These results are the first demonstration that estrogen-DNA adducts in urine are potential biomarkers for risk of developing breast cancer.

We continued to analyze medium from MCF-10F cells incubated with E_2 in Specific Aim 2. We detected estrogen metabolites, estrogen conjugates and the depurinating estrogen-DNA adducts 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua formed by MCF-10F cells. These results are described in several publications [2,3].

We analyzed mammary tissue and serum from ERKO/Wnt-1 mice and aromatase-transfected mice from Dr. Santen's laboratory (Specific Aim 4). We detected estrogen metabolites, conjugates and depurinating DNA adducts in the mammary tissue and serum from ovariectomized ERKO/Wnt-1 mice in a dose-related manner, depending on the dose of E_2 implanted into the mice. These results are described more completely in Specific Aim 4.

C. Key Research Accomplishments

We have established a procedure with the Acquity UPLC/QuattroMicro MS/MS to analyze 40 estrogen metabolites, GSH conjugates and depurinating DNA adducts with high sensitivity and selectivity.

We have successfully analyzed human nipple aspirate fluid and detected estrogen-DNA adducts in samples from women with breast cancer, but not in samples from women without breast cancer.

We have successfully analyzed human urine samples from women with and without breast cancer and at high risk for breast cancer and found that the level of depurinating estrogen-DNA adducts in urine from women with breast cancer and at high risk for breast cancer is significantly higher than the level of adducts in urine from healthy control women ($p<0.001$).

We have successfully analyzed human serum samples from healthy control women and found baseline levels of estrogen metabolites, conjugates and depurinating DNA adducts.

D. Reportable Outcomes

a. Publications

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c. Invited Presentations and Abstracts

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E. Conclusions

The Analytical Core has successfully implemented UPLC/MS/MS to detect estrogen metabolites, GSH conjugates and depurinating DNA adducts in human nipple aspirate fluid and urine from women with and without breast cancer and at high risk of developing breast cancer. The depurinating DNA adducts are present at higher levels in women with breast cancer and at high risk for breast cancer, compared to healthy control women. We are also analyzing estrogen metabolites, GSH conjugates and depurinating DNA adducts in human serum from healthy control women and will compare the levels with those in serum from women with breast cancer.

The Analytical Core is interacting with the other investigators in the Breast Cancer Center of Excellence to analyze a variety of samples for estrogen metabolites, estrogen GSH conjugates and depurinating estrogen-DNA adducts.

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ADVOCACY CORE - HART

Advocates have been and continue to be an integral part of this DoD COE grant, providing input into the specific aims of the grant as well as specific advocacy issues related to consent documents, pilot study design and implementation, and funding opportunities.

Specific projects outlined in the COE Advocacy Core:

Web-based model reference tool
The decision has been made to leave the web-based model reference tool under the auspices of the National Library of Medicine and remain available for consultation and input if they decide to incorporate our web-based tool as a part of their public information search tools.

Consumer Guide to Involvement in Basic Research
The consumer guide was published in 2007 and is available for upload to organizational websites as desired. This has been a labor of great satisfaction to the advocates of this COE and it is hoped that it will prove beneficial and interest more consumers in becoming involved in basic scientific research.

Article for publication based on current research
An article for publication continues to be in progress.

We continue to be very excited about the progress that is being made in prevention of cancer through this grant and the extraordinary findings outlined in this progress report. It is a privilege to be a part of such innovative research with these outstanding researchers who work collaborative and congenially and both welcome and value the participation of the advocates in this grant.